Trace Amine Associated Receptors: A New Target for Medications in Drug Addiction

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Abbreviations

5-HT	Serotonin
AEC	Animal Ethics Committee
AMP	Amphetamine
B-S	Bussey-Saksida
cAMP	cyclic adenosine monophosphate
DA	Dopamine
DAT	Dopamine transporter
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders
DST	Dorsal Striatum
g	Grams
GCPR	G-protein coupled receptor
i.p.	Intraperitoneal injection
METH	Methamphetamine
NAC	Nucleus Accumbens
NE	Norepinephrine
OFC	Orbito Frontal Cortex
PB	Phosphate Buffer
PBS-Tx	Phosphate Buffered Saline with Triton X
s	Seconds
s.c.	Subcutaneous injection
SSRI	Selective Serotonin Reuptake Inhibitor
TA	Trace amines
TAAR	Trace amine associated receptor
TAAR1	Trace Amine Associated Receptor 1
VTA	Ventral Tegmental Area

Abstract

The abuse of stimulant drugs, such as methamphetamine (METH), has become a major source of public concern in New Zealand. Specific medications for treating METH addiction are not available at present. The newly discovered trace amine-associated receptor 1 (TAAR1) constitutes a novel receptor target for medication development in neuropsychiatry. TAAR1 regulates monoamine systems in the brain, especially dopamine, and is activated directly by psychomotor stimulants, including METH. This study examined the effects of the newly developed TAAR1 partial agonist, RO5203648, in rat models of METH abuse. In experiment 1 rats were administered different doses of RO5203648 (0, 1.67, 5mg/kg i.p.) followed by METH (0, 0.75, 2mg/kg i.p.). Locomotor activity was monitored via automated video tracking system in an open field. The results revealed that RO5203648 dose-dependently reduced acute METH-induced stimulation and prevented long-term sensitization following chronic exposure. Paradoxically, in experiment 2, RO5203648 and METH treatment increased c-Fos protein expression in the nucleus accumbens and dorsal striatum. In experiment 3 rats were trained to consistently self-administer METH (0.5mg/kg/infusion) and were then pre-treated with RO5203648 (0, 3, 10mg/kg i.p.). The data showed that RO5203648 drastically reduced METH intake. Next, RO5203648 was substituted (0.25, 0.5, 1.0 mg/kg/infusion) for METH in the same paradigm. Remarkably, RO5203648 exhibited no reinforcing efficacy compared with METH. Taken together, these observations showed that RO5203648 is able to attenuate METH-related behaviours, including locomotor stimulation, sensitization and self-administration, and highlight the great potential of TAAR1-based medications for the treatment of METH addiction.

1. Introduction

1.1.Definitions: drug addiction, tolerance, withdrawal, and drug addiction cycle

Addiction and dependence are terms that are often used interchangeably to describe the same chronic relapsing disorder. For example, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, 2000) does not incorporate the term "addiction" but instead refers to "substance dependence" to denote the persistent and compulsive use of drugs despite problems related to use of the substance (DSM-IV, 2000). Addiction is an allostatic state in the brain reward system that acts through new reward set points. It involves a dysregulation of brain and hormonal stress responses, activation and recruitment of these responses and operation of the brain's learning and memory systems (Koob & Le Moal, 2008). Important factors of dependence include increased dosage amounts that progress to the use of high doses. This process is usually gradual and described as the development of tolerance. The amount of time a person has been using the drug is also important. Individuals who suffer from addiction experience a state of psychic and/or physical dependence on a drug (natural or synthetic) that is either consumed periodically or continuously. Characteristics of such a state span 3 domains; compulsion to obtain the drug, loss of control over the dose taken and the manifestation of a negative state when the drug is discontinued (withdrawal). This vicious cycle from drug use to abuse to addiction can also be viewed as a progression from positive to negative reinforcement as well as impulsivity to compulsivity (Eddy, Halbach, Isbell & Seevers, 1965; Koob, 2009). The DSM-IV (2000) criteria for substance dependence describes a cluster of cognitive, behavioural and physiological symptoms indicating that the individual continues to use the substance despite significant substance related problems. The

criteria for substance dependence and the cycle of addiction are depicted in *Figure 1* below (DMS-IV, 2000).

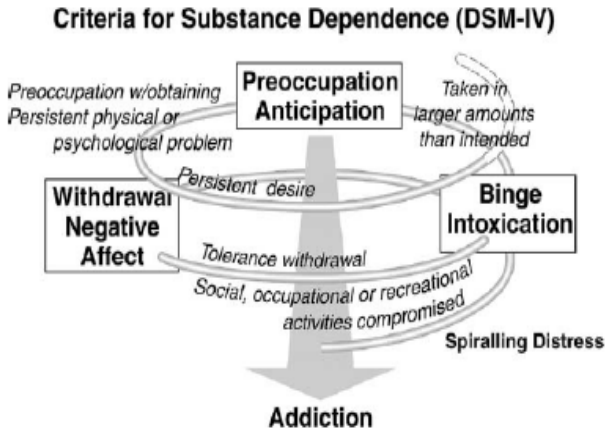


Figure 1: This diagram depicts a psychiatric perspective involving spiralling distress into addiction which consists of 3 main stages. Stage 1; preoccupation anticipation, stage 2; binge intoxication, and stage 3; withdrawal negative affect. Source: Koob & Le Moal, 2000.

The development of tolerance and withdrawal are key indicators that an individual has succumbed to drug dependence. Tolerance can develop rapidly and involves higher doses of the drug being needed to sustain the drug's positive effects (Julien, 2001). This is the result of a decrease in the number of receptors and a decrease in sensitivity and responsiveness of the target cell where the receptor is located. These drug receptors in tolerance are not permanently fixed therefore the system is acting homeostatically in an attempt to return toward the normal state.

Withdrawal is also homeostatic and is responsible for the negative reinforcement associated with continued drug use. Once an individual stops taking a drug, negative symptoms appear that typically characterize the exact opposite effect of the drug when under the influence. Symptoms can last for days or weeks depending

on individual's history with the drug. These symptoms can only be relieved by taking more of the drug and thus continuing the cycle of drug addiction (Julien, 2001).

It has long been conceptualized that impulse and compulsive control disorders both play a role in the development of drug addiction. Impulse control disorders are characterised by enhanced internal state of tension or arousal before an individual commits an impulsive act. At the time the act is committed there are subjective feelings of pleasure, gratification or relief (positive reinforcement). However, this may be followed by regret, self reproach or guilt. On the other hand, compulsive disorders are characterised by recurrent and persistent thoughts about the act and an individual will normally feel anxiety and stress before committing the act. Once the compulsive behaviour has been completed the individual experiences a relief from the stress (negative reinforcement). Koob et al (2004) suggests that as an individual transitions from an impulsive disorder to a compulsive disorder there is a shift from positive reinforcement to negative reinforcement driving the motivated behaviour (see *Figure 2*).

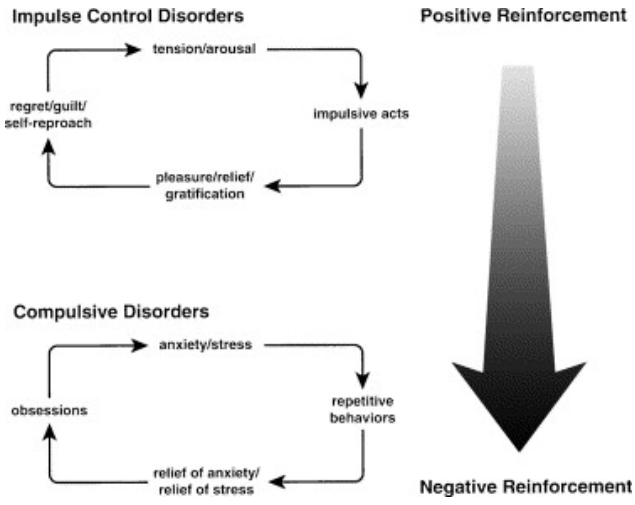


Figure 2: This diagram shows the stages of impulse control disorder and compulsive disorder cycles and the sources of reinforcement. Impulse control disorders are associated with positive reinforcement

due to the pleasure/gratification after the impulsive act whereas compulsive control disorders are more closely related to negative reinforcement because there is relief after the compulsive act. Source: Koob et al., 2004.

1.2.Behavioural theories of addiction: causes and maintenance of addiction

1.2.1. Opponent process theory

The opponent process theory first described by Solomon and Corbit (1974) proposes a novel way of observing motivation, in relation to addiction. This theory postulates that drug taking behaviour changes from positive reinforcement (euphoric state) to negative reinforcement (dysphoric state). This change occurs because homeostatic functions of reward fail to return to normal when under the influence of a substance, but the system is constantly trying to return back to a normal state thus exhibiting the reward dysfunction typical of addiction. This involves the concepts of an "a process" and a "b process". The "a process" accounts for the initial pleasant state that is activated in the mesolimbic dopamine (DA) projection to the nucleus accumbens (NAC) and amygdala, whereas the "b process" accounts for the negative withdrawal state that is activated via the hypothalamic pituitary adrenal (HPA) stress system. An "a state" is achieved when the pleasant state is reached via down regulation in the mesolimbic DA system. The "b state" occurs when the unpleasant state (from withdrawal) causes DA and serotonin (5-HT) levels to drop below normal. This drop may account for feelings of dysphoria when the drug is discontinued (Solomon & Corbit, 1974; Robinson & Berridge, 2003; Koob & Le Moal, 2008; Koob, 2009). The initial drug effect and subsequent addiction effects of both the "a state" and "b state" in the opponent process theory are depicted in *Figure 3*.

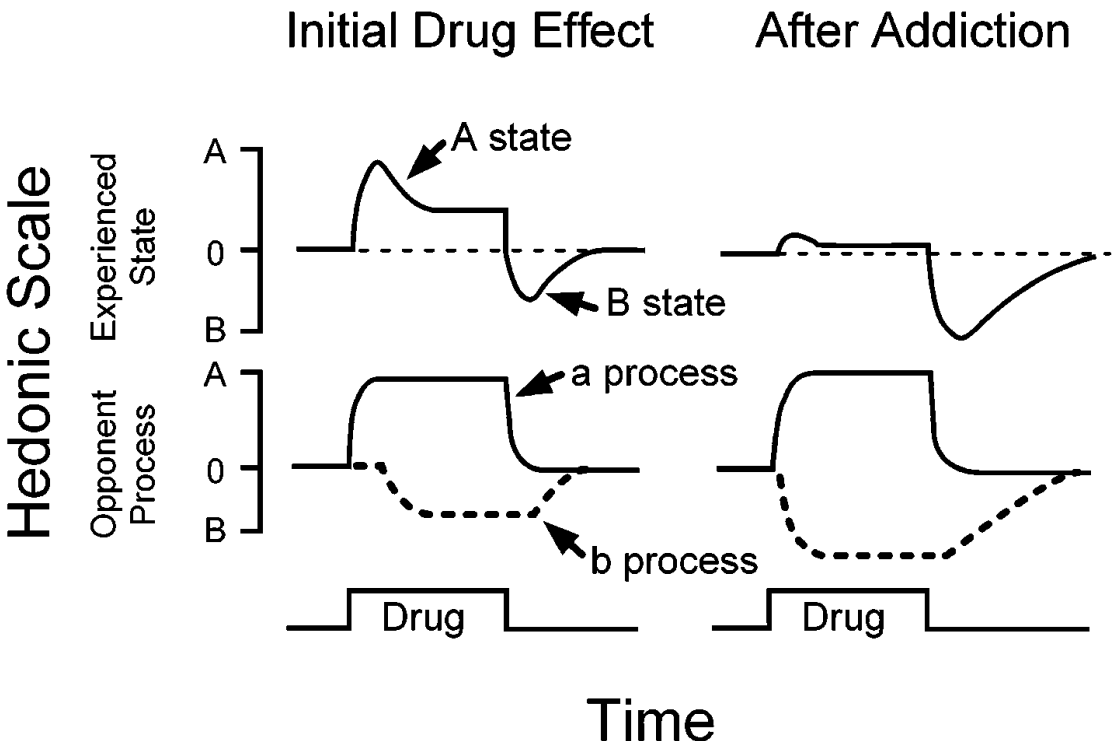


Figure 3: This diagram depicts the opponent process model of addiction, which involves an "a" state and a "b" state. The hedonic response to a stimulus (drug) is the "a-process" which elicits the "b-process". These processes combine to cause an initial pleasant "a-state" which is followed by an unpleasant "b-state". In the beginning of drug use the "a-state" experienced is large and the "b-state" is small. However, after repeated drug use the "b-process" becomes the dominate experience which typically involves the unpleasant symptoms of withdrawal. Source: Robinson & Berridge 2003.

1.2.2. *Allostasis*

This theory shares similar aspects of the opponent process theory. Allostasis is defined as the process of maintaining stability in regulating brain reward systems (Koob & Le Moal, 1997). Koob (2000) suggests that drug taking behaviour changes the parameters of the physiological systems in order to maintain stability within the brain reward systems. The change from normal reward set points to drug reward set points is seen as an allostatic state which effectively describes the reward dysfunction seen in drug addiction (Vanderschuren & Everitt, 2005). This theory implies that not

only brain functions change but also that whole body systems change in order to facilitate new set points induced by drug exposure. Chronic exposure to a drug may produce long-lasting abnormal functioning which may result in an individual being unable to respond adaptively to additional changes (Koob & Le Moal, 2000). *Figure 4* illustrates allostatic states when a drug is introduced. This theory is similar to the opponent process theory where "a" and "b" states are attempting to adapt to the changes a drug places on the body, physically and mentally.

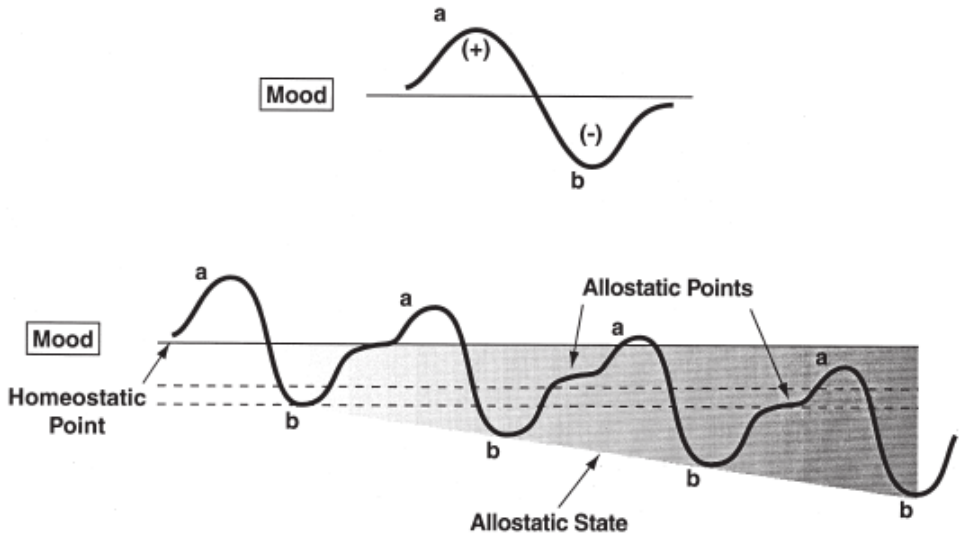


Figure 4: This diagram demonstrates an adaptation from Solomon and Corbit's (1974) opponent-process model of motivation to an allostasis theory of addiction. At the beginning of drug use there is a pleasant state "a-state" followed by an unpleasant state "b-state" and as drug use continues these states become unbalanced and produces a negative "b-state" that dominates the response to the drug. Source: Koob & Le Moal, 2000.

1.2.3. Incentive salience and sensitization

Incentive salience in relation to drug addiction explains several aspects of changes in behaviour through conditioned reinforcement, incentive motivation, behavioural sensitization and maladaptive stimulus response learning. Tiffany (1990)

proposed that after many cycles of drug taking, behaviour becomes dominated by drug seeking activities which lead to an automatic process. These drug seeking behaviours are triggered automatically by drug associated stimuli rendering individuals insensitive to the reduction in the drug reward. However this process does not lead to compulsion but rather it subverts neural mechanisms of stimulus-response habit learning causing maladaptive forms of habitual behaviour (Vanderschuren & Everitt, 2005). A similar theory, the learning hypothesis of addiction, has been suggested by Everitt and Robbins (1999). This theory states that addiction involves a transition from response-outcome to habit-like (stimulus-response) behaviour. This transition is mediated by information that transfers from the ventral striatum (NAC) to the dorsal striatum (DST) (Everitt & Robbins, 1999). Taken together these theories suggest that addiction is the result of implicit learning.

Sensitization

Sensitization can be defined as a progressive and enduring enhancement of a drugs effect. This phenomenon can be seen as the opposite of tolerance as the drug shows increased effects rather than diminished effects after repeated administration. Sensitization normally develops after frequent but intermittent administration whereas tolerance commonly develops after prolonged continued use of a drug. Thus, the development of tolerance is more commonly seen than sensitization (Lett, 1989; Szumlinski et al., 2000; Carlson, 2010; Xu et al, 2011). In 1993 Robinson and Berridge presented a theory of addiction known as the incentive sensitization theory. This theory postulates that individuals become addicted to drugs through Pavlovian learning processes where the motivational effects of the drug and the drug associated stimuli act as drug cues with incentive salience. This creates a change in neurons and

circuits involved in motivated behaviour where attention processing becomes biased towards drug associated stimuli, resulting in a compulsion to take the drug. These changes persist even after discontinuation from the drug, making the person more susceptible to relapse (Robinson and Berridge, 1993; 2000; Wyvell & Berridge, 2001; Vanderschuren & Pierce, 2009). More recently, Robinson and Berridge (2000) revisited the incentive sensitization theory of addiction and further examined animal models of craving. They stated that there are 4 basic points when considering the mechanisms behind drug addiction; 1) drugs with potential addictive ability produce lasting neural changes 2) those neural changes are involved in motivation and reward 3) neural systems become sensitized to drugs and their stimuli, and 4) neural systems mediate craving and wanting of the drug (Robinson & Berridge, 2000). *Figure 5* shows a representation of incentive sensitization in addiction.

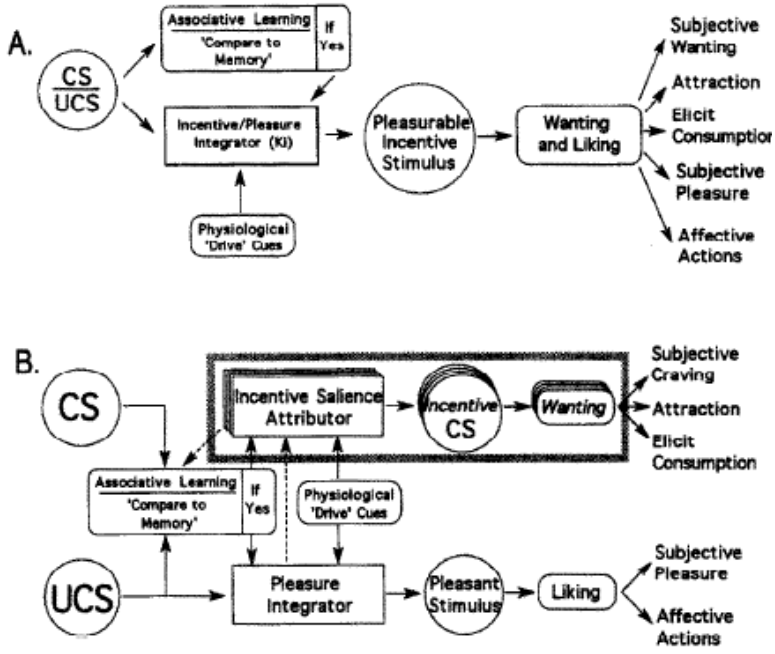


Figure 5: This complex diagram illustrates the incentive sensitization theory. (A.) demonstrates the model from which Robinson and Berridge adapt their theory of incentive sensitization which is seen below in (B.). The conditioned stimulus (CS) and unconditioned stimulus (UCS) are seen as attractive

and pleasant. Previous experience is associated with this pleasant state from memory and triggers physiological states (e.g. euphoria (positive) or withdrawal (negative)) that results in a "wanting" or "liking". Robinson and Berridge (1993) proposes that there are different psychological processes for "wanting" and "liking" a drug that is responsible for incentive salience. Source: Robinson & Berridge, 1993.

1.3 Neurobiology of addiction and the role of dopamine

As drug addiction develops several changes in the brains reward systems occur. Drugs of abuse seem to activate the same reward structures of the brain however specific drugs enter the common circuit at different points. These reward structures include projections from the Ventral Tegmental Area (VTA) to the basal forebrain which consists of the NAC, olfactory tubercle, amygdala and the frontal and limbic cortices. Also part of this system is the opioid peptide neurons within these connections. Projections from the VTA to the NAC may be implicated in the reward system for all drugs of abuse (Vanyukov et al., 2003).

The reward systems refer to the mesolimbic and mesocortical pathways, which are often called the mesocorticolimbic pathway. The neurons of the mesolimbic system are located in the VTA and project their axons to several parts of the limbic system, including the NAC, amygdala and hippocampus, all of which are relevant to drug reward, drug-related memories and conditioned responses (Volkow, Fowler & Wang, 2002; Carlson, 2010). The neurons of the mesocortical pathway are also located in the VTA and their axons project to the prefrontal cortex, including the cingulated gyrus and orbitofrontal cortex (OFC). *Figure 6* shows several structures and pathways that play a role in the effects of drugs of abuse. Dysfunction of these areas associates with the compulsivity of drug taking, poor inhibitory control, impaired incentive motivation and attribution of emotional valence (Volkow et al,

2002; Carlson, 2010). Both pathways involve dopaminergic neurons and their projections to several forebrain structures (Koob, 2009; 2009).

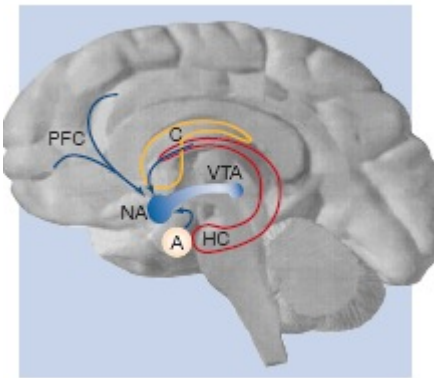


Figure 6: This diagram illustrates the pathways affected in drugs of abuse. VTA projects its neurons to several areas of the brain such as HC, NA, and A which all play a role in the reinforcing effects of drugs. Prefrontal cortex (PFC), Ventral tegmental area (VTA), nucleus accumbens (NA), amygdala (A), hippocampus (HC), and caudate nucleus or striatum (C). Source: Everitt & Robbins, 1999.

DA is an endogenous chemical belonging to a family of transmitters called monoamines, which includes other chemicals such as norepinephrine (NE), epinephrine and 5-HT. Their molecular structures are all similar and most addictive drugs, to an extent, affect their activity. A key factor in a drug of abuse's reinforcing effect is the ability to increase the concentration of the DA in the NAC. Some drugs (e.g. methamphetamine (METH) or amphetamine (AMP)) stimulate the release of DA in different brain regions, while other substances (e.g. cocaine) inhibit the reuptake of the monoamine serving as potent DA agonists (Julien, 2001).

The first stage of the addiction cycle, the binge/intoxication phase, are thought to involve the NAC-amygdala reward system, DA inputs from the VTA, local opioid peptide circuits, and opioid peptide inputs to the hypothalamus. Further, in the second sate of the addiction cycle, withdrawal/negative effects have been associated with

decreases in function of the extended amygdala reward system and recruitment of neurocircuitry related to stress. This involves an increase release of corticotrophin-releasing factor (CRF) in the amygdala, which has implications for anxiogenic and stress-like responses during the withdrawal stage (Weiss et al., 2001). The final stage of the addiction cycle, the preoccupation/anticipation phase, involves key afferent projections to the NAC and specifically to the prefrontal cortex (for drug induced reinstatement) and the basolateral amygdala (for cue induced reinstatement) (Koob, 2009). These structures help explain the mechanisms behind an individual's experience with drugs of abuse (e.g. positive reinforcement and withdrawal).

1.4 Methamphetamine

This study will focus on a particular drug of abuse, METH. METH is a highly addictive and potent psychostimulant drug that is compulsively abused. There are several different forms of METH; the most common among users are "speed", the powder form, "base", the oily paste form, and "ice" the crystallized form. There are also various methods of administering METH which include intravenous injection, smoking, snorting, and oral or anal ingestion. The most effective and immediate route of administration is intravenous injection or smoking, as the drug is directly absorbed into the blood stream or rapidly absorbed from the lungs, respectively. Snorting and oral ingestion can take 5 to 20 minutes to be effective (Molitor, Truax, Ruiz, & Sun, 1998; Julien, 2001; Rawson Conzales, & Brethen, 2002; Topp, Degnhardt, Kaye, & Darke 2002; Ciccarone, 2004; Cartier, Farabee, & Prendergast, 2006; Wu, Pilowsky, Schlenger, & Galvin, 2007). METH creates pleasurable experiences through a subjective increase in energy and elevated self-esteem as well as heightening sexuality, intensifying emotions, and impairing judgments (Strakowski, 1995; Molitor

et al, 1998; Rawson et al 2002; Cartier et al 2006). The immediate physiological changes when an individual uses METH are comparable to the fight or flight response which causes an increase in blood pressure, body temperature, heart rate, and breathing rate (Rawson et al, 2002). There is also a constriction of blood vessels and cardiac arrhythmia (Greenwell & Brecht, 2003). The euphoric state and effects can last up to 8 hours due to METH's slow metabolizing rate and subsequent long half life (Julien, 2001; Cartier et al, 2006). Once an individual stops using METH they will most likely experience a withdrawal syndrome, the effects of which are opposite to those it imposes. For example, loss of appetite can become increased appetite and weight gain, increased energy may become decreased and lead to fatigue, and elevated self-esteem and mood may develop into depression. Tolerance to the drug normally develops rapidly resulting in the administration of ever increasing doses to avoid the onset of the withdrawal syndrome (Julien, 2001).

1.4.1. Chemical description of Methamphetamine

METH (N-methylamphetamine), a lipophilic molecule, belongs to the phenylethylamine class of psychostimulants and is derived from AMP through the addition of a methyl (-CH3) group (see *Figure 7(a)*). Although both drugs attach to the same receptors, METH best fits this receptor and elicits a more powerful response from the cell (Julien, 2001). In other words METH has a much quicker distribution into the central nervous system (CNS) and crosses the blood brain barrier quicker than AMP. METH falls into a group of drugs known as "releasers" (pp172. Riddle, Fleckenstein, & Hanson, 2008) therefore it reallocates DA into the cytoplasm and as a result DA levels rise and DA is released through reverse transport (see *Figure 7 (b)*). This process leads to a significant increase in synaptic DA levels (Barr, et al., 2006;

Riddle et al, 2008). The release of synthesized DA induced by METH increases the amount of DA available to the postsynaptic receptor which causes the reinforcing effects of the drug (Vearrier, Greenberg, Miller, Okaneku, & Haggerty, 2012). As well as altering DA levels, METH also affects serotonergic, noradrenergic and glutamatergic systems causing neurotransmitter dysregulation. However, long term use of METH or even high doses of METH can produce decreases in the concentration of DA and 5-HT as well as a decrease in DA transporter (DAT) activity. There is also damage to the dopaminergic system due to the neurotoxic effects METH exerts on the brain (Julien, 2001; Riddle et al, 2008; Ares-Santos et al., 2011). METH also alters the expression of early immediate genes such as cFos, which is used as a marker of neuronal activation. METH induces cFos expression in brain regions known for their role in drug reward, including the striatum and prefrontal cortex. These systems include dopaminergic and serotonergic pathways and projections (Thiriet, Zwiller, & Ali, 2001). Several studies have documented a dose-dependent increase in cFos expression after METH (Umino Nishikawa, & Takahashi, 1995; Thiriet et al, 2001) and cocaine administration (Chocyk, Czyrak, & Wedzony, 2008). Thus cFos expression provides a useful marker for the effects of such substances (Umino et al, 1995).

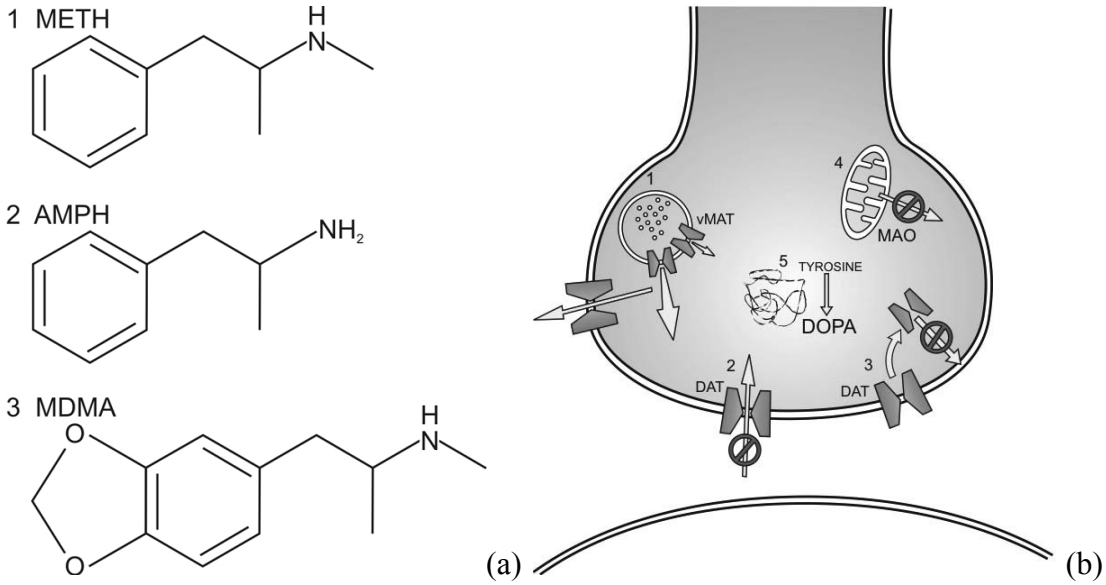


Figure 7: (a) 1. Chemical structure of METH and from which it is derived 2. AMP, as well as 3. MDMA (b) Neurobiological mechanisms of METH at the synaptic level. 1. Redistribution of catecholamine from synaptic vesicles to the cyotsol 2. Reverse transport of neurotransmitters through the DAT. 3. Decrease of DAT. 4. Inhibition of MAO thereby increasing cytosolic levels of monoamines. 5. Increase of expression and activity of tyrosine hydroxylase. Source: Barr et al., 2006.

1.4.2. Methamphetamine prevalence and related issues

Several studies have examined the prevalence of illicit drug use in New Zealand and abroad finding an alarmingly high level of drug use and abuse (Boden, Fergusson, & Horwood, 2006; NZADU, 2011; UNODC, World Drug Report, 2010). The New Zealand Alcohol and Drug Use Survey conducted from 2007 to 2008 reported that the use for all types of amphetamines, including METH, was at a prevalence of 3% for the general population. Data from the Christchurch Health and Development Study (Boden et al., 2006) reported that 3.6% of the sample by the age of 25 met the criteria for dependence on illicit drugs. 11.9% had reported using stimulants, including cocaine, AMP, and METH. A common finding in these studies

was that those using AMP-like drugs were more likely to be young, male, and of Maori ethnicity (Boden et al, 2006; NZADU, 2011).

METH's impact on society is largely seen through emergency department influx of trauma, mental health problems and long term health issues (Eddy et al 1965, Richards et al., 1998). Negative psychiatric, affective and physical symptoms are also frequently observed in METH users not only because they are at risk of developing METH-induced psychosis but as a drug using population they are more likely to suffer from schizophrenia and other psychotic disorders (McKetin, McLaren, Lubman, & Hides, 2006). Psychiatric symptoms include paranoia, auditory/visual hallucinations and delusions of persecution (Topp et al, 2002; McKetin et al, 2006). Affective symptoms are manifested through depression, mood swings and aggression that typically escalate to violence (Topp et al, 2002). Depression and suicidal ideation is frequently reported in this population. For instance, 16% of youth METH users reported having suicidal ideation. Suicide attempts whilst under the influence of METH are also very common. Further support for these claims comes from evidence that METH and alcohol are the most common substances found in the blood of individuals who have committed suicide (Richards et al, 1998). In a sample of METH users 13% screened positive for psychosis and 23% exhibited 1 psychotic symptom. Psychosis has been reported 11 times higher in METH users compared to the general population (McKetin et al, 2006).

Physical symptoms consist of disturbed or disrupted appetite and sleep patterns, skin problems likely resulting from stereotypical "pick/grooming" behaviour, decreased immunity to infection, high body temperature, stomach cramps and shaking (Topp et al, 2002; Rawson et al, 2002). A survey found that 38% of

emergency department visits were for METH-related chest pains and 28% were METH intoxicated patients. Emergency department visits related to stereotypical skin picking accounted for 6 % of patients and 54% of those were admitted to hospital (Vearrier et al, 2011). Dental decay is another general result of METH use that is commonly referred to as "meth mouth" and is associated with lack of oral hygiene and malnutrition. Contractions of diseases such as HIV, Hepatitis A, B and C, and Sexually Transmitted Diseases (STDs) are also highly likely in METH users due to risky sexual behaviour and/or use of contaminated needles when administering the drug intravenously (Molitor et al, 1998; McKetin et al, 2006; Vearrier et al, 2011).

These negative side effects can often lead to more serious health problems commonly seen in chronic users of METH. This includes but is not limited to severe paranoia and lasting psychosis, deterioration of judgment and fine motor skills, malnutrition, exhaustion and systemic or soft tissue infection from injecting (Richards et al, 1998). Stroke, cardiac value thickening, pulmonary hypertension, and decreases in lung functioning can also be seen in long term users of the drug (Julien 2001; Greenwell & Brecht, 2002). METH use can also cause intracranial haemorrhage, the most devastating neurological side effect of consuming the drug. Other common neurological side effects include ischemic stroke, seizures and cognitive impairment that may be persistent even when abstaining from the drug. METH can also be fatal, directly or indirectly, commonly due to neurological and cardiac complications and trauma (Vearrier et al, 2011).

Another pressing concern is that the use of METH by pregnant women is becoming more common resulting in adverse pregnancy outcomes and perinatal maternal death. Adverse pregnancy outcomes include; foetal growth restriction,

premature delivery, placental insufficiency, and haemorrhage, as well as putting the foetus at risk of intraventricular haemorrhage and cavitary lesions in the brain. These adverse outcomes cause subsequent problems in the child's life such as poor performance on attention and verbal memory tests (Rawson et al, 2002; Vearrier et al, 2011).

There are also various dangers from clandestine METH labs which are hazardous to the people involved and the general population. The risks include blast injuries, thermal burns, chemical injuries and toxic exposures. The standard of METH made in clandestine labs is often low and contains substantial impurities that are even more harmful to the user than pharmaceutical grade METH. Other substances are often added into illegally made METH to enhance quantity and prices (Vearrier et al, 2011).

METH-related violence, crime and recidivism rates are also increasing. Vearrier et al (2011) reported that homicide accounted for 27% of METH-related deaths. A recent law change that addresses the issue of METH use and abuse is the Combat Methamphetamine Epidemic Act of 2005, where products containing pseudoephedrine, ephedrine and phenylpropanolamine are restricted and regulated as these have the key ingredients to manufacture METH (Vearrier et al, 2011). This is one of the many ways government and other communities are trying to reduce the accessibility of METH and METH-related substances (Cartier et al 2006).

1.5 Medications used to treat methamphetamine dependence

Effective pharmacological treatments of stimulant addiction remain limited and scarce. Currently there are no medications available that are approved by the

Food and Drug Administration (FDA, US) for the specific treatment of psychostimulant dependence. Various antidepressant medications such as Monoamine Oxidase Inhibitors (MAOI's) and Selective Serotonin Reuptake Inhibitors (SSRI's) have been examined as potential treatments of substance use disorders. However, despite the co-morbidity of addiction and depressive symptoms, the efficacies of such treatments are low. Other pharmacological treatments for substances of abuse have only had limited success with nicotine and heroin such as varenicline (Chantix) (Berlin, 2011) and the methadone program, respectively (Ciccarone, 2011).

The American Academy of Paediatrics and American College of Obstetrics and Gynaecology recommend screening at risk populations who are abusing drugs and therefore at risk for developing addiction. Non-pharmacological approaches towards addiction include early screening that focuses on risk reduction, client centred counselling and setting treatment goals. Other behavioural and social approaches involve cognitive behavioural therapy (CBT), community reinforcement approach and contingency management. All treatments aim to change maladaptive behaviour through learning strategies and increasing coping skills to prevent relapse and by providing positive reinforcement such as money, goods, or natural social support for non-drug-related behaviour (Ciccarone, 2011).

Animal studies investigating potential treatment of METH and other psychostimulants using SSRIs and other mood altering drugs are abundant. Szumlinski, Balogum, Maisonneuve, & Glick (2000) investigated ibogaine (IBO), a recognized anti-addictive agent, and 18-methoxycoronaridine (18-MC), a synthetic iboga alkaloid congener, on METH-induced behavioural sensitization. This was measured through locomotor activity and stereotypy. Results showed that pre-

treatment of 18-MC enhanced locomotor activity in rats sensitized to METH. Both 18-MC and IBO augmented the expression of stereotypy. These findings suggest that pre-treatments with iboga agents do not exhibit anti-addiction properties in rats chronically exposed to METH (Szumlinski et al., 2000).

A later study by Shuto et al (2006) investigated the DA D_1 receptor agonist, R-(+)-SKF38393 (SKF), and its ability to reverse behavioural sensitization to METH. METH-induced behavioural sensitization was measured through locomotor activity. At the neural level, DA levels in the striatum were measured through microdialysis. METH-induced behavioural sensitization was observed as increased locomotor activity to subsequent drug challenge. After a 7 day treatment with SKF, METH-induced increased locomotor activity was reversed by the DA D_1 receptor agonist. Microdialysis also revealed reduced DA release in the striatum after a METH challenge. However these results did not hold true for cocaine studies. Ideally, possible treatments for psychostimulant addiction would be able to reduce the effects of several drugs in this group. These findings suggest a possible therapeutic approach to psychostimulant addiction based on DA D_1 receptors (Shuto et al, 2006).

Higley et al (2011) further explored DA receptors and evaluated the selective DA D_3 receptor antagonist, SB-277011A, as a potential medication for psychostimulant addiction. A previous study had already demonstrated the antagonist's efficacy in reducing cocaine self-administration and reinstatement (Xi et al., 2005). Similarly, this more recent study examined SB-277011A in animal models of METH addiction through self-administration and reinstatement. Results showed that SB-277011A did not affect METH self-administration. However, under a progressive ratio SB-277011A was able to significantly reduce the break point,

demonstrating an ability to reduce METH-reinforcing efficacy. The DA antagonist was also successful in reducing METH-induced reinstatement (Higley et al, 2011).

Another possible treatment approach to psychostimulant dependence is the newly developed *N*-substituted benztropine analogues (BZT). These molecules exhibit potential for becoming a substitute medication through their high affinity to the DAT and ability to block DA uptake. Ferragud et al (2009) tested the BZT derivative 3s-[bis(4-flurorphenyl)methoxy]-tropane (AHN-1055) on cocaine addiction in locomotor activity and self-administration paradigms. Results revealed that AHN-1055 had some stimulant properties including increased locomotor activity. However, the BZT analogue was able to reduce cocaine self-administration and did not exhibit strong reinforcing effects when given in a self-administration paradigm. A similar study using the same BZT derivative AHN-1055 by Velazquez-Sanchez, Ferragud, Renau-Piqueras, & Canales (2010) investigated AMP addiction using conditioned place preference (CPP), locomotor activity, sensitization, self-administration and DeltaFosB accumulation in the NAC. Their findings indicated that AHN-1055 attenuated AMP-induced behaviours in the CPP and locomotor activity assays. AHN-1055 was also found to block behavioural sensitization to AMP and successfully diminished AMP self-administration. Analysis of DeltaFosB in the NAC revealed reduced expression of the protein after repeated administration of AHN-1055. After AMP conditioning the BZT analogue produced a drastic reduction in DeltaFosB accumulation. These findings support the development of DA uptake blockers with low abuse liability as a possible treatment for psychostimulant addiction.

Another newly developed BZT derivate, JHW007, has also been tested for its potential therapeutic effects on stimulant addiction. Velazquez-Sanchez, Ferragud,

Murga, Carda, & Canales (2010) examined the effects of JHW007 in cocaine models of addiction in mice. This was measured in CPP and the elevated plus maze assays. JHW007 did not exhibit place conditioning when administered alone, however, the analogue was able to block cocaine-induced conditioned preference. The BZT derivative did not stimulate locomotor activity either, yet it was able to dose-dependently reduce cocaine-induced locomotor stimulation and prevent sensitization. Results from the elevated plus maze again demonstrated JHW007's ability to reduce locomotor activity. Further, JHW007 treatment did exhibit anxiogenic-like effects in the elevated plus maze (Velazquez-Sanchez et al., 2010).

The generalization from animal studies to human conditions is difficult, especially when developing new therapeutic approaches to psychostimulant addiction. Shoptaw et al (2008) conducted a randomised, placebo-controlled trial of bupropion, a DA and NE reuptake inhibitor, for the possible treatment of METH dependence. This involved a 12-week trial period where 73 participants seeking treatment for METH dependence were given either bupropion or a placebo, alongside contingency management and CBT sessions. Participant's progress was monitored 3 times a week using a battery of measures including the Addiction Severity Index-lite (ASL), the Beck Depression Inventory (BDI), and also through urine samples. Unfortunately, there were no significant results between bupropion and placebo groups suggesting the treatment was unsuccessful in treating METH dependence. However, there was a significant reduction in cigarette smoking associated with bupropion, supporting its use as a smoking cessation drug. This implies bupropion may not be a METH dependence medication (Shoptaw et al, 2008).

A later study investigating modafinil, a non amphetamine-type stimulant, for treatment of METH dependence was assessed using a randomised, double-blind, placebo-controlled design (Heinzerling et al, 2010). Previous animal studies had already supported its use for treatment in METH relapse (Yahyavi-Firouz-Abadi & See, 2009). Due to modafinil's stimulant-like profile it was thought that this drug could improve withdrawal symptoms from METH dependence similar to the methadone treatment programme for heroin users. This trial lasted 12 weeks where 71 participants received either modafinil or a placebo. Additionally, contingency management and CBT sessions were implemented and measures were taken 3 times a week using the same methods as Shoptaw et al (2008). Results showed no significant effects of modafinil in METH dependence compared to the placebo group. Unlike bupropion, modafinil did not reduce cigarette smoking but it did not increase smoking either (Heinzerling et al, 2010). These findings suggest modafinil does not exhibit therapeutic-like effects in the human population. Both bupropion and modafinil showed no significant effects for treating METH dependence in humans, thus the hunt continues for a successful medication.

Grant, Odlaug and Kim (2010) explored the treatment efficacy of an opioid antagonist, naltrexone, and a glutamate modulator, N-acetyl cysteine for METH dependence. Over the course of the 8 week pilot study several measures were taken from 31 participants. These measures included the Penn Craving Scale, frequency of METH use, urine toxicology, the clinical global impression (severity) scale, the Hamilton rating scale for depression and anxiety, the Sheehan disability scale, and the quality of life inventory scale. Results showed that the effects of the combination of naltrexone and N-acetyl cysteine treatment were similar to the effects of the placebo.

It was reported that there were no improvements in METH craving or use in the active treatment group (Grant et al, 2010). A review investigating vaccines for drug abuse and dependence suggests that naltrexone is an effective pharmacotherapy for opioid relapse however this does not hold true when given to psychostimulant abusers (Shen, Orson, & Kosten, 2012).

Zorick, Sugar, Hellemann, Shoptaw, & London (2011) examined the SSRI, sertraline, and its effects on METH-dependent research participants. This placebo-controlled trial compared participants who had METH-positive urine tests to their pre-randomization baseline. Results showed that those treated with sertraline had increased METH intake compared to those who were in the placebo treatment group (Zorick et al, 2011). These results demonstrated that SSRIs and other antidepressant medication treatments are ineffective in psychostimulant addiction. These studies have only been partially successful or are in need of further research to fully understand the mechanisms behind psychostimulant addiction.

1.6 Development of new medications to treat methamphetamine dependence: Trace Amine-Associated Receptors

The recent discovery of trace amine associated receptors (TAARs) holds promise for a potential treatment in stimulant addiction. TAARs are a class of endogenous amine compounds that overlap with classic biogenic amines such as 5-HT, noradrenaline, adrenaline, DA and histamine. These classical biogenic amines play significant roles in numerous functions in the brain such as hormone regulation and motor control. They are also significantly implicated in emotional/cognitive functions and neurotoxicity, and have been involved in the aetiology of depression, attention deficit/hyperactivity disorder, schizophrenia, Parkinson's disease and drug

addiction (Bradaia et al., 2009). Receptors activated by monoamines resulting from amino acid metabolism include p-tyramine, octopamine, tryptamine and B-phenylethylamine, all of which are commonly referred to as trace amines (TAs). TAs such as these are metabolites of amino acids that are found at low concentrations in the brain (Lindemann & Hoener 2005; Bradaia et al, 2009; Xie & Miller 2009; Revel et al., 2011).

In the last decade research has shown that TAs bind to members of the G-protein coupled receptor (GCPR) family. TAs that bind to GCPRs-associated receptors are referred to TAARs. Since their discovery several TAARs have been identified, however only TAAR1 and TAAR4 have been found to respond to typical TAs. TAAR4 has little or no expression in the brain and is only present in the olfactory epithelium, whereas TAAR1 is found in brain monoaminergic regions and is co-expressed in a subset of DA neurons along with the DAT (Borowsky et al., 2001; Bradaia et al, 2009).

TAAR1 signals through G proteins to elevate intracellular cyclic adenosine monophosphate (cAMP) levels in response to TAs. TAAR1 may also regulate DA transmission by inhibiting DA uptake and enhancing DA efflux through the DAT. Expression of TAAR1 was reported to be moderate in several monoaminergic cell groups including the substantia nigra, VTA, locus coeruleus and dorsal raphe. Whereas low levels of expression were present in other regions of the brain such as the amygdala and cerebellum. Therefore TAAR1 may be important in sub serving psychostimulant action, as it can be activated by not only TAs but also by psychostimulant drugs (Bunzow et al., 2001; Grandy, 2007; Xie & Miller, 2009).

Overlap of TAAR1 distribution with TA binding sites in the monoaminergic systems further suggests that they may recognize endogenous brain TAs and mediate

their function. TAs may be able to activate TAAR1 in locations such as the synaptic cleft, even if extracellular levels of TAs are low, which may be important under certain pathological or pharmacological conditions, including drug-taking and addiction. Revel et al (2011) found that TAAR1 control monoamine driven behaviours and suggested anxiolytic-like and antipsychotic-like properties for TAAR1 agonists such as R05166017. This selective TAAR1 agonist was also shown to reduce the frequency of 5-HT neuron firing in the dorsal raphe, suggesting that TAAR1 may also modulate the serotonergic system, which may have implications for the process of addiction.

There is some evidence that specifically links TAAR1 with psychostimulant-related phenomena. For example, Xie and Miller (2007) found that TAAR1 is activated by both DA and METH. Their study also demonstrated that METH causes a TAAR1-dependent inhibition of DA uptake and DA release through the DAT. Further, Wolinsky et al (2007) showed that TAAR1 knock-out mice had deficits in prepulse inhibition (which are also induced by psychomotor stimulants) and enhanced sensitivity to AMP, thus supporting a role for TAAR1 in mediating the effects of stimulant drugs. These findings provide a basis for furthering research using TAAR1-related drugs as treatment for neuropsychiatric disorders such as addiction.

1.7 Aims and Hypotheses of the Study

The present study examined the newly developed TAAR1 partial agonist, RO5203648, and its effects on METH-induced behaviours. Adult male rats were exposed to METH and the RO5203648 compound in different drug addiction paradigms and immunoreactive assays including locomotor activity, sensitization,

self-administration, and cFos expression. It was hypothesised that the RO5203648 would have attenuating effects on METH-induced behaviours. To date there is no research exploring the TAAR1 partial agonist RO5203648 in models of METH addiction. Therefore the present study is the first step towards examining and developing a potential pharmacological medication against METH addiction based on TAAR1 mechanisms.

Experiment 1(a) investigated the efficacy of the partial agonist, RO5203648, in a model of METH-induced locomotor behaviour. Locomotor activity is an accurate measure of drug-induced behaviour (Fujiwara, Kazahaya, Nakashima, Sato, & Otsuki, 1987; Cunningham, Finn, & Kelley, 1997; De Vries, Schoffelmeer, Binnerkade, Mulder, & Vanderschuren, 1998; Szumlinski et al., 2000; Kuczenski & Segal, 2002; Shuto et al., 2006; Hall, Stanis, Avila, & Gulley, 2008; Velazquez-Sanchez et al., 2010; Xu et al., 2011). It was hypothesised that animals treated with the partial agonist would show a reduction in METH-induced locomotor activity in both acute and chronic stages of treatment. Experiment 1(b) examined RO5203648's ability to attenuate long-term sensitization to METH. Sensitization is known to develop shortly after repeated intermittent administration of a drug (Strakowski, Sax, Setters, & Keck, 1996; Stahl et al., 1997; Cunningham et al., 1997; De Vries et al., 1998; Wyvell & Berridge, 2001; Kuczenski & Segal, 2002; Nelson & Killcross, 2006; Hall et al., 2008; Velazquez-Sanchez et al., 2010; Xu et al., 2011).It was predicted that animals treated with RO5203648 would not exhibit sensitization after chronic exposure to METH. In experiment 1(c) RO5203648 and METH were examined in a stimulus-response paradigm to investigate the effects the partial agonist has on habit learning. It was predicted that the RO5203648 would reduce habit formed behaviour in rats

previously exposed to METH. Lastly, in experiment 1(d) the partial agonist's effects on impulsivity was also measured and it was hypothesised that the TAAR1 agonist would have attenuating effects on impulsivity after exposure to METH.

Experiment 2 (a) investigated the acute effects of METH and RO5203648 on locomotor stimulation. It was predicted that pharmacological treatments would have the same effects as seen in experiment 1(a), in that RO5203648 would attenuate METH-induced locomotor activity. Experiment 2 (b) examined the induction of the immediate early gene cFos in the NAC and DST following exposure to METH and RO5203648. It was predicted that METH treated rats would show an increase in the expression of cFos (Umino et al, 1995; Thiriet et al, 2001) and that this effect would be attenuated in rats pre-treated with RO5302648.

Experiment 3 (a) examined RO5203648 in a METH self-administration paradigm. It was hypothesised that a pre-treatment with the partial agonist would reduce METH self-administration and therefore its reinforcing effects. Experiment 3 (b) evaluated the partial agonist's abuse liability also in the self-administration context. It was theorized that RO5203648 would not have reinforcing effects and animals would not be motivated to self-administer the TAAR1 partial agonist. Lastly, in experiment 3(c) RO5203648 was tested for its effects on responding for natural reward with a prediction that RO5203648 would not alter motivation to seek natural reinforcement.

2. Materials and Methods

2.1. Subjects and housing conditions

A total of 71 male PVG hooded rats were bred in house at the animal lab of the psychology building at the University of Canterbury (Christchurch, New Zealand). In addition, 16 male Long Evans rats were sourced from the University of Otago (Dunedin, New Zealand). All rats were housed in a temperature and humidity controlled colony room with a reversed 12 hour light/dark cycle (lights off 08:00-20:00). Rats were kept in groups of 4 per opaque plastic cage (50 cm x 30 cm x 23 cm) standard housing conditions. Water was given ad libitum at all times throughout experiments. All experiments were approved by Animal Ethics Committee (AEC) at the University of Canterbury.

2.1.1. Experiment 1

54 male PVG hooded rats were randomly allocated to 1 of 9 experimental groups split into a 3 x 3 design each receiving differing doses of METH and the TAAR1 agonist (see Table 1). All rats were approximately 8 months old and weighed between 350 - 380 g. During this experiment, 2 rats died resulting in 5 or 6 rats per group as detailed in Table 1.

Table 1: Shows the experimental groups using a 3x3 design. Group 1 was a control group who received a pre-treatment of saline followed by a second dose of saline. Group 2 was a low METH control group who received a pre-treatment of saline and a small dose of METH (0.75mg/kg). Group 3 was a high METH control group who received a pre-treatment of saline and a high dose of METH (2mg/kg). Group 4 was a control group TAAR1 agonist who received a small dose of the TAAR1 partial agonist, RO5203648 (1.67mg/kg) followed by an injection of saline. Group 5 was an experimental group for the low doses of the TAAR1 partial agonist (1.67mg/kg) and METH (0.75mg/kg). Group 6 was an experimental group for the low dose RO5203648 (1.67mg/kg) and the high dose of METH (2mg/kg). Group 7 was a control group for the high dose of RO5203648 (5mg/kg) followed by an injection of saline. Group 8 was an experimental group for the high dose of RO5203648 (5mg/kg) and the low dose of METH (0.75mg/kg). Group 9 was an experimental group for the high doses of RO5203648 (5mg/kg) and METH (2mg/kg)

Group (No. of Rats)	RO5203648 Doses (mg/kg)	METH Doses (mg/kg)
1 (5)	0	0
2 (5)	0	0.75
3 (6)	0	2
4 (6)	1.66	0
5 (6)	1.66	0.75
6 (6)	1.66	2
7 (6)	5	0
8 (6)	5	0.75
9 (6)	5	2

2.1.2. Experiment 2

17 Male PVG hooded rats were randomly assigned into 1 of 4 groups receiving treatments of RO5302648 and/or METH as detailed below in Table 2. All rats received food and water ad libitum and their weight was recorded weekly. All rats for this experiment were approximately 16 months old with an average weight range of 360 – 420 g.

Table 2: Shows the experimental groups where group 1 was a control group that received an i.p. injection of 20% DMSO with saline followed by another injection of saline. Group 2 received an injection of 20% DMSO with saline followed by a dose of METH (0.75mg/kg). Group 3 received a dose of RO5203648 (5mg/kg) and saline. Group 4 was administered a combination of RO5203648 (5mg/kg) and METH (0.75mg/kg).

Group (No. of Rats)	RO5203648 Doses (mg/kg)	METH Doses (mg/kg)
1 (4)	0	0
2 (4)	0	0.75
3 (4)	5	0
4 (5)	5	0.75

2.1.3. Experiment 3

The self-administration experiments used 16 male Long Evans (6 Long Evans rats were subsequently lost and therefore did not complete all self-administration experiments due to health related complications, only some associated with surgery). Animals were randomly allocated into experimental (10) or control groups (6). The experimental group was trained to press for METH reinforcements whereas the control group was trained to press for saline reinforcements. All rats for this experiment were approximately 3 months old and weighed an average of 350 - 450 g.

2.2. Pharmacological treatments

Methamphetamine hydrochloride was obtained from BDG Synthesis (Wellington, New Zealand) and dissolved in 0.9% physiological saline. RO5203648 (partial TAAR1 agonist) was donated by Hoffman-La Roche LTD (Switzerland) and dissolved in 0.9% physiological saline and either 10% or 20% DMSO. All compounds

were injected intraperitoneally (i.p.) at 1 ml/kg with doses administered on the basis of body weight. Drug doses are expressed as milligrams per kilogram (mg/kg).

2.3. Behavioural apparatuses

2.3.1. Open field apparatus

4 identical black Perspex open field boxes (50 cm x 40 cm x 35 cm) were used and monitored with a video tracking system and image analysis software (Viewpoint 2.5, Champagne au Mont D'Or, France) that provides automatic measures of travelled distance, trajectory and velocity of the subjects. All open fields used were placed on the floor in a windowless room (see appendix A). The experimenter was not present in the room during testing.

2.3.2. Impulsivity and Stimulus Response Apparatus

Rats were tested in 8 separate Bussey-Saksida (B-S) Touch screen Chambers (Campden and Lafayette Instrument Co, UK). These consisted of a sound attenuating chamber with a touch screen at 1 end and a reward trough at the other (see appendix B). The touch screens were run by 2 Pentium computers (1 for 4 chambers) with ABET II Touch Paradigms computer software which recorded key aspects of the rats' behaviour including responses and latencies. Chambers were also equipped with a pellet dispenser that released 45 mg chocolate flavoured sucrose pellets (Bioserve, New Jersey, USA). All testing was conducted in a windowless room with the experimenter not present in the room during testing.

2.3.3. Self-administration apparatus

8 light and sound attenuating operant conditioning chambers were used to train and test the rats for self-administration (see appendix C). These chambers were

equipped with 2 response levers, a self-administration tube, a house light and a stimulus light (Panlab, S L, Barcelona, Spain). Each lever press was recorded as a response either active or inactive depending on the protocol. The operant conditioning chambers were controlled by computers using the Packwin software package. All testing was conducted in a windowless room with the experimenter not present in the room during testing.

2.4. Self-administration surgery

All rats were anaesthetized with Avertin (2,2,2-tribromoethanol, 12.5mg/ml, in 2.5% tertiary amyl alcohol, 2ml/100g of body weight) i.p. and Carprofen was administered before surgery as pain relief (5mg/kg i.p.). Catheters (O/D 0.63 mm, I/D 0.30 mm, Camcaths Cambridge, UK) were implanted into the right jugular vein, exiting dorsally between the scapulae. Rats were kept warm during surgery by the heat of the overhead lamps and thick layers of sheet underneath them. The rat's condition was monitored throughout surgery and post surgery. Analgesic cream was applied to the back and neck incision areas following suturing. To prevent infection rats were treated post surgically with daily injections of antibiotic (Cephalexin, 10mg/kg s.c.) for 7 days. Researchers and laboratory technicians monitored all rat's recovery with post surgery checklists (see appendix D). Before testing animals were given a full week to recover, and more time was given if incisions were slow to heal. Catheters were flushed with heparinised saline (0.1 ml, 70IU/ml) before and after each self-administration session. All surgical procedures were carried out under aseptic conditions and in compliance with the University of Canterbury AEC guidelines as well as being subject to AEC approval.

2.5. Immunohistochemistry and microscopy

2.5.1. Perfusions

All rats from experiment 2 were deeply anesthetized with sodium pentobarbitone (300mg/ml) and perfused transcardially first with 150 ml of chilled saline followed by 150ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were post-fixed in 4% paraformaldehyde for a minimum of 24 hours and then transferred to long-term solution (20% glycerol in a phosphate buffered solution containing 0.5% sodium Azide) and stored at 4 degrees Celsius for a minimum of 48 hours or until the brain had sunk to the bottom of the container.

2.5.2. Tissue preparation

Brains were cut in 40 µm coronal sections on a sliding microtome with a freezing block (Thermofisher, Germany). Coronal sections were obtained through each of the following regions: NAC and DST. Sections were taken from +3.70 mm to -0.26 mm from Bregma. Each slice was placed in a vial filled with a 0.1 M phosphate buffered solution. Approximately 4 copies were taken of each section (i.e. 4 vials each containing 1 in every fourth slice)

2.5.3. Immunohistochemistry cFos

Free floating sections (1 vial per rat) were rinsed several times in 0.1M phosphate buffered saline containing 0.3% Triton X-100 (PBS-Tx) and treated with 0.3% H_20_2 in PBS-Tx to inhibit endogenous peroxidise. The sections were incubated overnight with gentle rotation at 4 degrees Celsius in a Fos rabbit polyclonal primary antibody (Santa Cruz, CA, USA) diluted at 1:1000 in PBS-Tx. The sections were rinsed several times the following day with PBS-Tx and then incubated for 1 hour

with a biotinulated goat anti rabbit secondary antibody diluted in 1:1000 PBS-Tx. After several more rinses in PBS-Tx the sections were incubated for 1 hour in ABC+NGS in PBS-Tx. Once removed from the avotin biotin complex the sections were rinsed again in PBS-Tx while the visualizing solution was being prepared. This consisted of 0.1 M PB solution with 0.001% diaminobenzidine and Nickel (II) sulphate added to produce a nuclear black reaction. This solution was filtered twice to remove any precipitate and at the last minute 3.3microl H_2O_2 was added. The tissue sections were submerged in the visualising solution for 5 minutes before being removed and washed several times with PB to stop the reaction. Sections were mounted from distilled water onto subbed slides and allowed to dry overnight at room temperature before cover slipping. Mounted slides were dehydrated with graduated alcohol dips 70%, 95% and 100% and then cleared in xylene. Cover slips were then fixed with DPX mounting medium and left to dry overnight.

2.5.4. Microscopy

High resolution photomicrographs of the NAC and DST were taken at 10X magnification with an optical microscope (Nikon Eclipse E800). Digital photographs were taken and analysed with image analysis software (Image Tool, UTHSCSA, USA). To obtain an average density counts were taken from four consecutive sections in both hemispheres of the NAC and DST. The position of the frame remained constant in the NAC using the anterior commissure as a reference. Threshold intensity was controlled manually within a constant range to eliminate background stain. Positive cFos cells were counted and the results were expressed as cell density (cells/mm^2) in each of the regions studied.

2.6. **Statistical analysis**

All data was analyzed with repeated measures analysis of variance (ANOVA) and *post-hoc* Scheffe's tests were also conducted. All data was processed using the Statview 5.0 statistical programme and presented in graphical form. An alpha of $p<.05$ was used for all statistical analyses.

3. Experimental Procedures

3.1. Experiment 1

3.1.1. Food Deprivation and reward habituation

All 54 rats were placed on food deprivation where they received 12-15 g of standard rat chow/rat/day before pre-training began. Individual weight was kept at 90% of their free feeding weight which was recorded before the start of their diet. This was to ensure rats were adequately motivated to perform the task. Animals were also habituated to the food reward that was to be used in the task by placing small chocolate flavoured pellets into the rat's home cages each day for 7 days before the start of pre-training. After pre-training rats were given food ad libitum over the course of pharmacological treatments and open field testing (14 days). Once open field testing was completed rats were placed back onto food deprivation in order to perform the stimulus-response habit learning and impulsivity tasks. All rats received water ad libitum throughout the experiment.

3.1.2. Bussey-Saksida pre-training and habituation

In order to habituate the animals, rats were pre-trained initially to eat 5 chocolate pellets presented in the food tray in the Bussey-Saksida (B-S) touch screen chambers. 8 rats, 1 in each chamber, were placed in to the boxes with the chocolate pellets already in the food tray. These sessions lasted 30 minutes each or until the pellets were eaten. Once all rats were consistently eating the chocolate pellets from the feed tray manual hand-shaping began. For this rats received reinforcement for being in the half of the chamber closest to the touch screen. Through successive approximation the rats were hand-shaped to receive reinforcement only when they

showed interest in the touch screen. Overnight sessions using an auto-shaping protocol were used to ensure rats learnt the behaviour in addition to the 1 hour sessions during the day. During the auto-shaping the touch screens presented a stimulus in the centre with 2 keys on either side. Both keys in the auto-shaping protocol were reinforced with 1 food pellet. Unfortunately, a side bias occurred in most of the rats therefore an un-biasing task was developed and rats were trained on this for several days until the bias had been eliminated. The un-biasing protocol required the rat to nose poke both keys in the B-S chambers. If 1 key was pressed 15 times it became inactive. The protocol finished when 30 reinforcements had been received or when 20 minutes had elapsed. Once all rats were nose poking consistently in the B-S chambers, habituation and testing in the open field boxes began.

3.1.3. (a) Open Field testing (Locomotor Activity)

All rats were habituated to the open field boxes with 2 concurrent 30 minute sessions over 2 days. Over the duration of 14 days 4 rats were tested concurrently in 4 separate open fields. RO5203648 treatments (0, 1.67, 5 mg/kg, i.p.) were injected 10 minutes before METH treatments (0, 0.75, 2 mg/kg i.p.). Immediately after the injections of METH rats were placed into the open fields and behaviour was recorded for 60 minutes.

3.1.4. (b) Open Field Testing (Sensitization)

After locomotor testing was complete rats were placed back on food deprivation (12-15 g/rat/day) and were withdrawn from all pharmacological treatments for 3 days. On the fourth day all rats, regardless of group, were given a low

dose of METH (0.25 mg/kg) as a sensitization probe and placed back in the open fields in order to test for sensitization effects of METH.

3.1.5. (c) Stimulus-response (habit learning)

Once all open field testing was completed the rats were returned to the B-S touch screen chambers for a stimulus-response task. For this task a stimulus (either a cross or a circle) was presented in the centre of the screen. The rat was then presented with 2 keys 1 on the left and 1 on the right. If a cross was presented then the right key would be positively reinforced with a food pellet and if a circle was presented then the left key would be positively reinforced with a food pellet. If the rat nose poked the right key when a circle was presented then there would be no consequences and vice versa for the left key and cross. A nose-poke at the reinforced stimuli and correct key was considered a correct response and resulted in a food pellet being dispensed. Correct and incorrect responses were recorded on the touch screen chambers computer software. Each session consisted of 60 trials.

3.1.6. (d) Impulsivity

After rats had completed the stimulus-response task or reached a predetermined cut off (+500 trials) without learning they were administered varying doses of METH 5 minutes before returning to the B-S chambers for impulsivity testing. 3 different doses of METH (0, 0.10, and 0.25 mg/kg) were tested with 3 different delays (0 s, 10 s, 30 s). Initially the rats completed a forced choice task where they learnt 1 key represented a large reward with or without a delay and the other key represented an immediate small reward. On completion rats were changed to the free choice impulsivity task where they could choose which key they prefer. This task ran for 25 trials. In half of the chambers the right key represented the large reward with delay

and the other half had the left key as the large reward with delay in order to counterbalance. Drug administration was also counterbalanced so that half of the rats were administered drugs and delays in ascending order and other half in descending order.

3.2. Experiment 2

3.2.1. *Food regulation*

All rats were given food and water ad libitum throughout this experiment.

3.2.2. Open field testing (locomotor activity)

Rats were habituated to the open fields for 1 hour the day before testing begun. 4 rats were tested concurrently in 4 separate open fields. Rats were allowed to pre-habituate for 5 minutes before the session started. Animals were administered RO5203648 or the vehicle (saline and 20% DMSO) 10 minutes prior to receiving the METH dose or saline equivalent. Immediately after receiving their second treatment rats were placed into the open field apparatus and their behaviour was recorded for 2 hours. Once behavioural testing was completed rats were removed and euthanized with pentobarbital for perfusion and later analyzed for immunochemistry and microscopy.

3.3. Experiment 3

3.3.1. Food maintenance

All rats in this experiment were placed on food maintenance and received rat chow at 15-18 g/rat/day. This was to ensure the rats were motivated to perform the task.

3.3.2. Self-administration training and testing

Before surgery rats were pre-trained to press a lever for saccharin (5% solution) reinforcement. After the surgery, the rats were trained on METH or saline self-administration under a fixed ratio (FR1) schedule. All rats had to reach a minimum of 15 reinforcements before testing began.

3.3.3. (a) METH or saline self-administration

Once rats showed 3 days of stable responding (with less than 20% variance), pre-treatments of RO5203648 through i.p administration were introduced at different doses of 0, 3, and 10 mg/kg. These were given in a randomised order 10 minutes before starting METH self-administration in the chambers. Testing occurred in operant boxes with 2 retractable levers serving as active and inactive levers in counterbalanced fashion. Each active lever press resulted in an illumination of a stimulus light for 5 s and a time out period of 20 s. An inactive lever press resulted in an error sound for 0.2 s and a time out of 20 s. Each session lasted 90 minutes. After the completion of all 3 different RO5203648 pre-treatment tests rats were made to return to stable responding under METH or saline reinforcement before moving onto the next phase of the experiment.

3.3.4. (b) RO5203648 substitution

Self-administration of the TAAR1 partial agonist was also tested for psychoactive properties. Varying doses of RO5203648 were given through intravenous infusions (0, 0.25, 0.5, and 1 mg/kg). Rats had to meet the criterion of 15+ reinforcements under METH or saline self-administration before being tested

again with a different dose of RO5203648 substitute dose. After all substitution doses of RO5203648 (0, 0.25, 0.5, 1.0 mg/kg/infusion) were obtained a small dose of METH was administered.

3.3.5. (c) Saccharin reinforcement test

Rats were placed back onto saccharin reinforcement to ensure RO5203648 did not affect their ability to respond for normal reward. After 2 sessions of stable responding (15+ reinforcements) rats were pre-treated with doses of RO5203648 (0, 3, 10 mg/kg i.p.) 10 minutes before the start of the saccharin self-administration session.

3.3.6. Catheter patency tests

These tests were done to ensure all catheters were working well once the rats had finished RO5203648 pre-treatment testing and before RO5203648 self-administration testing was conducted. This was done by infusing a small amount of pentobarbital (10 mg/kg/infusion) into the catheter, if the rat succumbed to the sedative effects of the pentobarbital it was concluded that the catheter was working effectively.

4. Results

3.1. Experiment 1 results

This experiment investigated RO5203648 effects on METH-induced behaviours including locomotor activity and sensitization. Cognitive effects of METH and RO5203648 were measured through stimulus-response and impulsivity tasks.

3.1.1. Locomotor Activity

It is well known that METH has the ability to increase locomotor activity (Fujiwara et al., 1987; Szumlinski et al., 2000; Shuto et al., 2006; Hall et al., 2008). To determine the effects of RO5203648 and its ability to modulate METH-stimulated locomotor activity, rats were treated during 14 consecutive days and the locomotion was measured every 2 days throughout the 2-week period. A repeated measures ANOVA was performed with treatment groups (METH and RO5203648) as the repeated measure and independent variable and locomotor activity for all sessions as the dependent variable. The results for the overall sessions indicated a significant effect of the treatment variable, $F(4,52) = 4.735$, $p = .0030$ (see *Figure 8* and Table 3*)*. There was a significant effect in METH treated groups, $F(2,52) = 36.367$, $p < .001$ and also in RO5203648 treated groups, with and without METH treatment, $F(2, 52) = 7.069$, $p = .0022$). RO5203648 dose-dependently reduced METH-induced locomotor activity as shown in *Figure 8*.

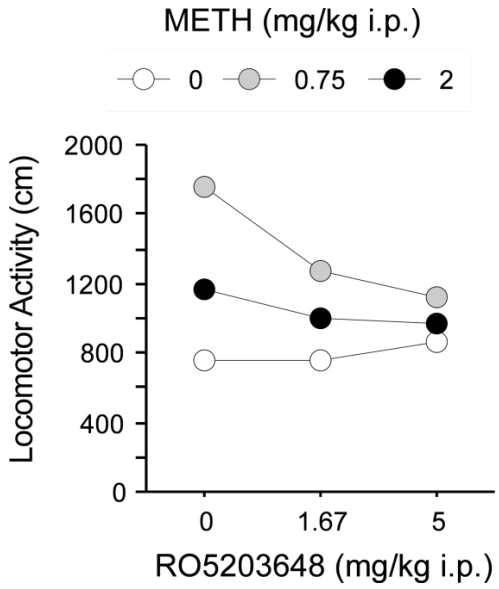


Figure 8: Interaction graph shows the locomotor activity mean deviation score (±SEM) across all groups receiving METH and RO5203648. There is a clear dose-dependent reduction in METH-induced locomotor activity.

Table 3: Mean, standard deviations (SD), and standard error (SE) corresponding to data points in *Figure 7* for all treatment groups for locomotor activity in overall sessions.

Treatment Groups	Mean	SD	SE
0-0	753.682	407.242	19.871
0-0.75	1760.706	475.406	23.197
0-2	1170.940	475.987	21.202
1.67-0	754.695	487.935	21.734
1.67-0.75	1272.817	370.149	16.488
1.67-2	1006.262	504.849	22.488
5-0	869.200	350.426	15.609
5-0.75	1124.958	393.404	17.524
5-2	972.428	471.797	21.016

Exploration of this interaction with *post-hoc* Scheffe's test revealed that, rats receiving METH, showed increased locomotor stimulation compared to saline treated rats. There were significant differences between the low dose of METH and saline (*M*

= -571.435, *SD* = 174.944, *p*<.001), and the high dose of METH and saline (*M* = -257.184, *SD* = 174.469, *p* = .0024). There was also a significant difference between both doses of METH (*M* = 314.251, *SD* = 174.469, *p*<.001). The low dose of METH (0.75mg/kg) elicited a higher rate of locomotor activity in relation to the high dose of METH (2mg/kg) shown in *Figure 8.* A Scheffe's test again revealed significant differences between the low dose of RO5203648 and saline treated rats (*M* = 213.591, *SD* = 177.251, *p* = .0146) and between the high dose of RO5203648 and saline (*M* = 237.987, *SD* =177.251, *p* = .0059). In addition there was no significant difference between the low and high dose of RO5203648 (*p* = .9374).

The average locomotor activity across all sessions showed a significant effect of the main treatment factor, *F*(6,52) = 3.215, *p* = .0046. There was also a significant interaction between session and METH treated groups, *F*(12,52) = 2.706, *p* = .0019 but not for RO5203648 and session. This means that the rats treated with METH showed significantly different rates of locomotor activity averaged across all sessions. This is demonstrated in *Figure 9* as the low dose of METH had significantly enhanced locomotor activity compared to the high dose of METH (see *Figure 9*).

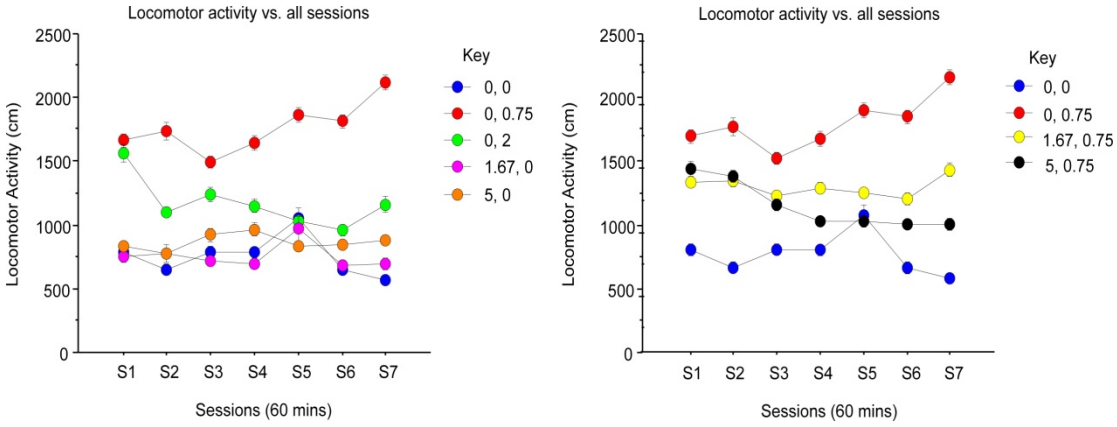


Figure 9: Mean deviation scores (±SEM) for locomotor activity across all sessions. The low and high doses of RO5203648 (1.67, 5 mg/kg i.p.) reduces METH-induced (0.75 mg/kg i.p.) locomotor activity. This difference becomes more apparent with each session. The low dose of METH (0.75 mg/kg i.p.)

exhibited increased locomotor activity compared to the high dose of METH (2 mg/kg i.p.). Locomotion for saline rats remains low throughout all sessions.

An interaction between time and all sessions showed a significant result, $F(66,52) = 3.459$, $p<.001$. Another significant result for a 3-way interaction across all Sessions x Time x METH, $F(132,52) = 1.423$, $p = .0014$, showed the increased rate of METH at the low dose compared to both the high dose of METH and saline. A Scheffe's test revealed a significant difference in average locomotor activity for all groups between the first session and sixth session ($M = 275.628$, $SD = 221.931$, $p = .0039$). This implies that the average locomotor activity significantly increased from session 1 to session 6 (see *Figure 9*).

Taken together, these findings clearly demonstrate that RO5203648 dose-dependently attenuates METH-stimulated locomotor activity in the rat without producing any significant effects when administered alone. Moreover, RO5203648's ability to reduce METH-induced locomotor activity became more apparent as chronic treatment progressed.

3.1.2. Sensitization

After the 14 day treatment period, rats were withdrawn from all pharmacological treatments for 3 days and then tested for a sensitized response to METH (0.25mg/kg). A repeated measures ANOVA with treatment groups as the independent variable and the METH probe as the dependent variable revealed a clear sensitization effect with rats previously treated with METH as shown in *Figure 10*. Both the high and low dose of METH showed enhanced locomotor activity compared to the saline group after all rats received the small dose of METH (0.25 mg/kg). There

was a significant interaction between METH and RO520348 when given the drug challenge, $F(4,52) = 3.041$, $p = .0271$ (see *Figure 10*).

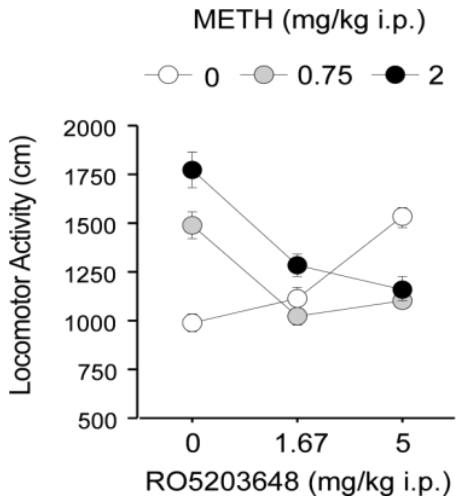


Figure 10: Locomotor activity mean deviation scores (±SEM) for RO5203648 and METH. This graph depicts the complex results for the METH probe test (0.25mg/kg i.p.) where RO5203648 appears to block METH-induced sensitization but also exhibits some cross sensitization with RO5203648 alone treatments

These data showed that chronic exposure to both moderate and high doses of METH induce long-term sensitized locomotor responses, as measured following administration of a probe METH treatment. Previous exposure to RO5203648, even at low doses, during the METH sensitizing regimen completely prevented METH sensitization. Furthermore, the data reveals the presence of cross sensitization between RO5203648 and METH, as rats exposed to the high dose of RO5203648 responded strongly to METH compared to animals previously exposed to saline or low dose RO5203648. It is important to note that RO5203648 prevented METH sensitization at doses (1.67 mg/kg) that did not cross-sensitize with METH.

3.1.3. Stimulus-response habit learning

To study potential cognitive effects induced by the chronic drug treatment, rats performed the stimulus-response task in the B-S chambers, after the 14-day treatment

period and completion of the sensitization test. Again, a repeated measures ANOVA was conducted with the treatment groups as the independent variable and the number of correct responses for the presented stimulus as the dependent variable. Despite extensive training (each rat performed more than 500 trials), rats were unable to effectively choose the correct responses regardless of treatment group. The average correct response rate was also just above chance; the average response was just over 30 out of 60 trials (see *Figure 11*).

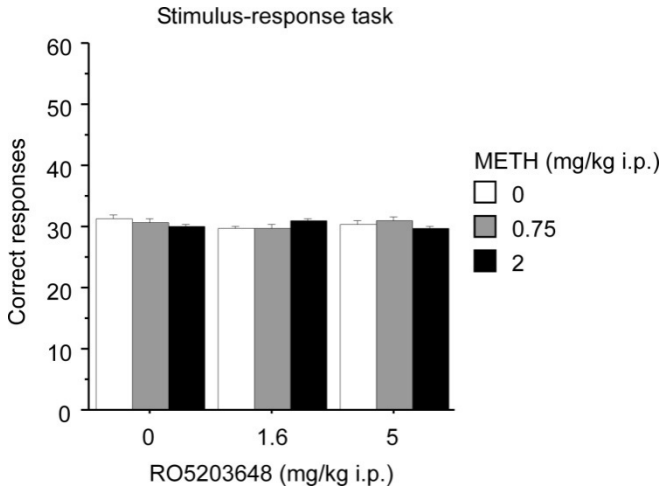


Figure 11: Shows the mean deviation scores (±SEM) for the interaction between RO5203648 and METH treatment groups in the stimulus response paradigm. This graph demonstrates lack of effect found in this task.

There was no significant results across sessions (p = .1455). Interactions between sessions and RO5203648 treatments were also not significant (p = .4771) as well as the interaction between session and METH treatments (p = .4283). A 3-way interaction between sessions RO5203648 treatment and METH treatment again was not significant (p = .1423). Further results showed no significant differences across groups and session times.

The lack of learning the stimulus-response task prevents drawing any conclusions in relation to the effects that chronic METH exposure may have on this parameter and, similarly, the effects of RO5203648 could not be determined.

3.1.4. Impulsivity

A task to measure impulsive behaviour was conducted after the 14 day treatment period, sensitization test and stimulus-response task. This was done to determine whether RO5203648 alone or its pre-treatment before METH had any effect on impulsive choice following treatment with low doses of METH (0, 0.1, 0.25mg/kg i.p.). This involved rats responding for a small reward with no delay and a large reward with 3 different delays (0 s, 10 s, and 30 s). A repeated measures ANOVA was performed with the treatment groups being the independent variable and the number of large responses (with or without delays) being the dependent variable. This analysis revealed no significant difference between treatment groups (p = .1146) and between the interaction treatment group x delayed response (p = .8609). However there was a significant result between delay responses, $F(2,52)=29.439$, $p<.001$ (see *Figure 12*). Scheffe's tests revealed significant difference between 0 s delay and both 10 s and 30 s delay ($p<.001$). These results imply that the impulsivity task was sensitive, as rats decreased responding for the larger reinforcement when delay was introduced, doing so in a delay-dependent manner. However, cognitive deficits as a result of chronic METH exposure were not evident.

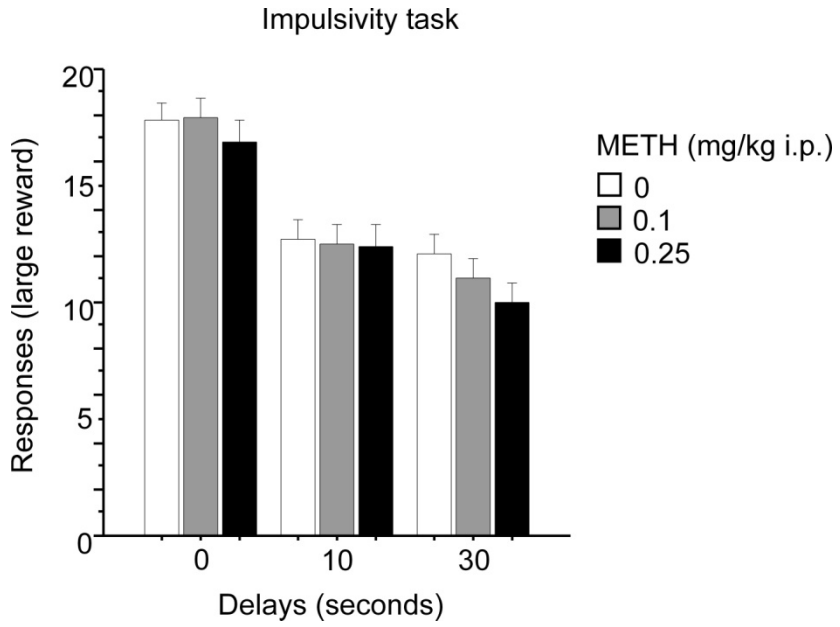


Figure 12: Mean deviation scores (±SEM) for the responses (large reward) for all conditions (delays vs METH probes). This graph shows there is no significant difference in responding between groups.

The effect of probe doses of METH was near significant but did not meet the cutoff (p = .0714) whereas the interaction effect of METH doses and treatment groups was not significant (p = .1420).

Overall, the impulsivity task did not reveal any significant effects of chronic METH exposure. Moreover, there were no clear effects of RO5203648 on METH-induced impulsive choice.

3.2.Experiment 2 Results

The aim of this experiment was to measure the acute effects of METH, RO5203648, its combination and saline after a single administration on locomotor activity and cFos expression. cFos protein expression was examined in the NAC and DST.

3.2.1. Locomotor Activity

With the aim to examine the neural mechanisms underlying the locomotor effects of METH, RO5203648, and their interaction, rats were exposed to either saline, METH

(0.75 mg/kg i.p.), RO5203648 (5 mg/kg) or their combination (METH, 0.75 mg/kg and RO5203648, 5 mg/kg i.p.) and locomotor activity was subsequently measured for 2 hours. An ANOVA was performed with treatment groups as the independent variable and the locomotor activity the dependent variable. The results showed a significant effect of treatment on locomotor activity, $F(4,4) = 193.668$, $p<.001$. *Post-hoc* tests revealed rats treated with METH alone or with the combination of METH and RO5203648 had significantly increased locomotor activity compared to the saline and RO5203648 alone treated groups (see *Figure 13*). It is important to note that RO5203648 was not able to reduce METH-stimulated locomotor activity. In experiment 1, RO5203648 was able to attenuate METH-induced locomotion by ca.15% following acute treatment, with chronic treatment of the compound being more effective.

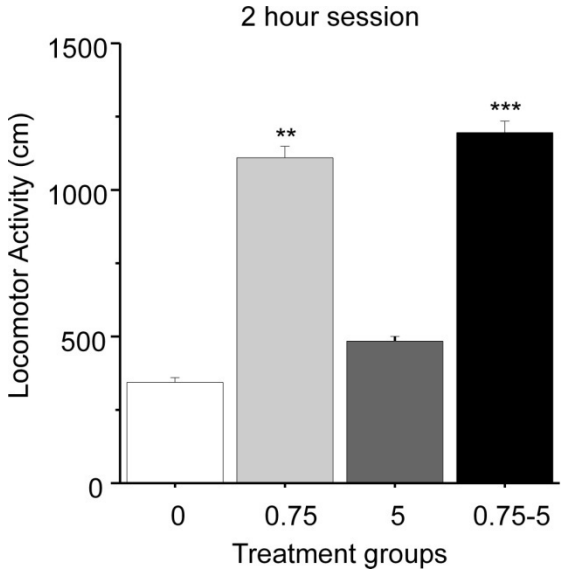


Figure 13: Mean deviation score (±SEM) for locomotor activity across all treatment groups. This graph demonstrates enhanced locomotor activity for both METH and the combination of RO5203648 and METH compared to RO5203648 alone and saline treated rats. *** indicates statistical significance of $p<.001$. ** indicates statistical significance of $p<.01$.

3.2.2. cFos expression in NAC and DST

A repeated measures ANOVA of the cFos expression data was conducted with the treatment groups as the independent variable and the cell count as the dependent variable. This analysis revealed a significant result for treatment effect, $F(3,4) = 14.994$, $p<.001$. There was also a significant result for cFos expression in both brain regions $F(1,4) = 8.759$, $p = .0111$. The interaction between cFos Count x Treatment was significant at $F(3,4) = 6.104$, $p = .0080$ (see *Figure 14* and *Figure 15*). *Post-hoc*, Scheffe's test revealed further significant results. The group administered the combination treatment of RO5203648 (5 mg/kg) and METH (0.75 mg/kg) had a significantly higher amount of cFos expression compared with RO5203648 alone ($M = -103.146$ $SD = 61.064$, $p = .0012$) and saline groups ($M = -113.248$, $SD = 61.064$, $p<.001$) for the overall cell count in NAC and DST. There was also a significant difference between the overall average cell count in the NAC compared to the DST (M = -32.433, SD = 21.343, p = .0059). However, there was no significant difference between rats treated with METH alone and saline ($p = .1076$), RO5203648 alone ($p = .2270$) or the combined treatment of RO5203648 and METH ($p = .0628$) for the overall expression of cFos in both brain regions analysed.

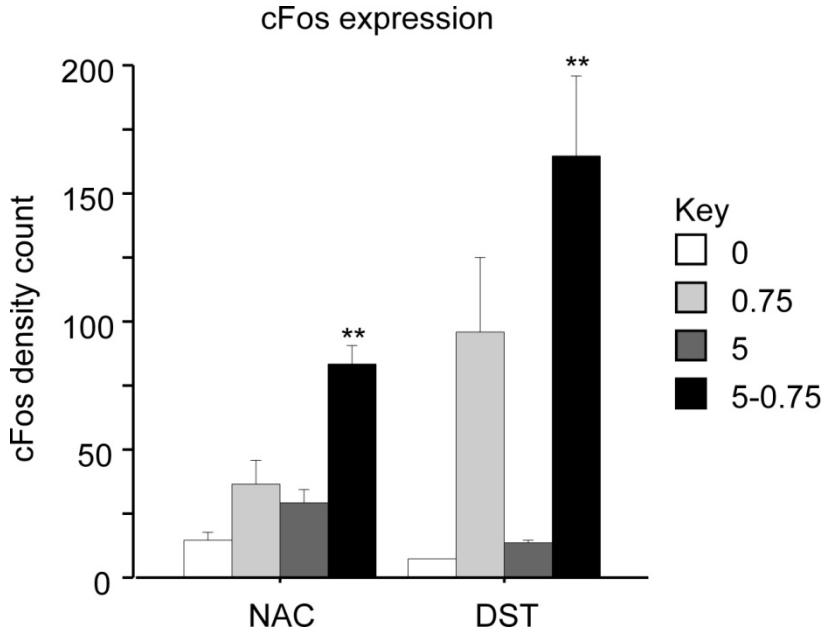
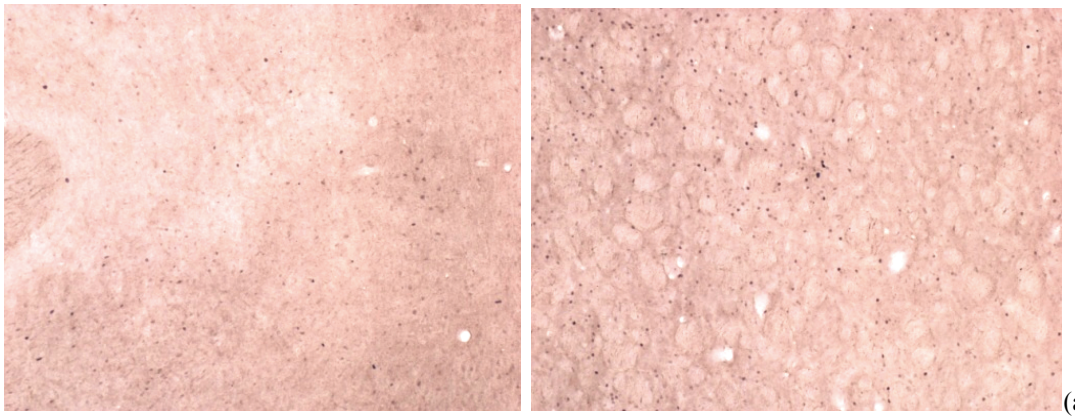
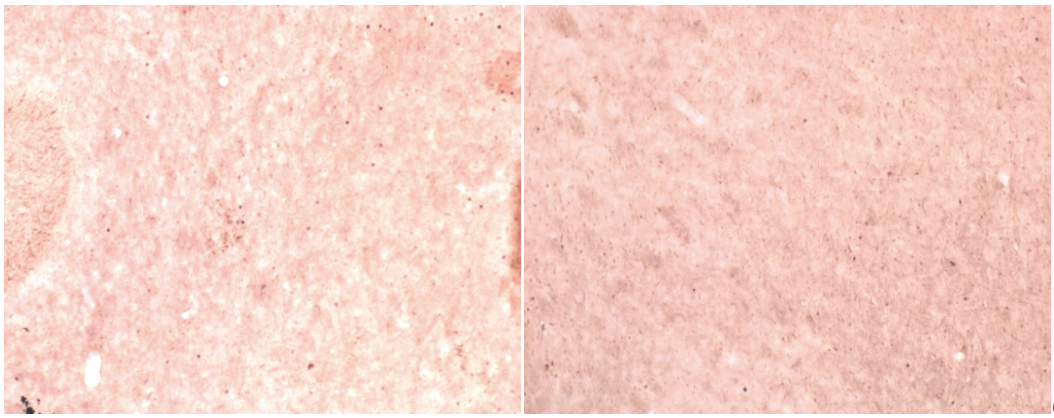


Figure 14: Mean deviation scores (±SEM) for both NAC and DST across all treatment groups. There is a significant increase in both the NAC and DST cFos expression for the group administered both RO5203648 and METH. ** indicates statistical significance of $p<.01$.



(a)



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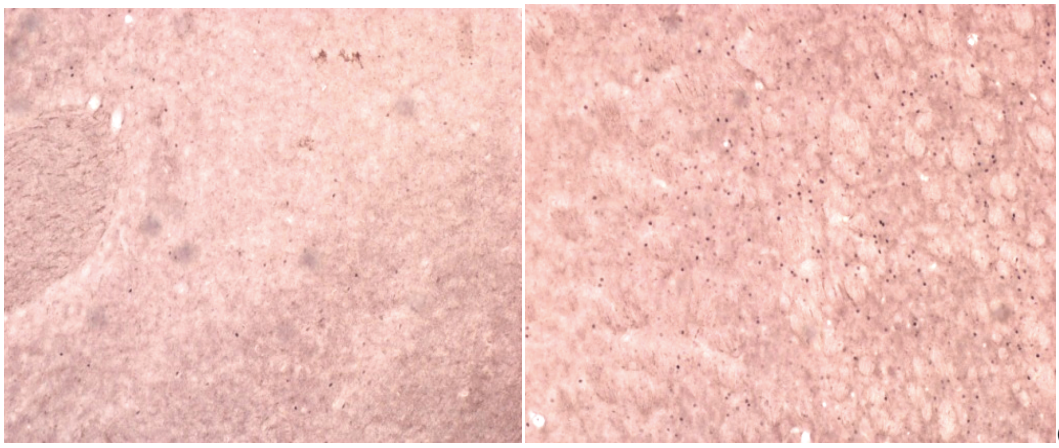

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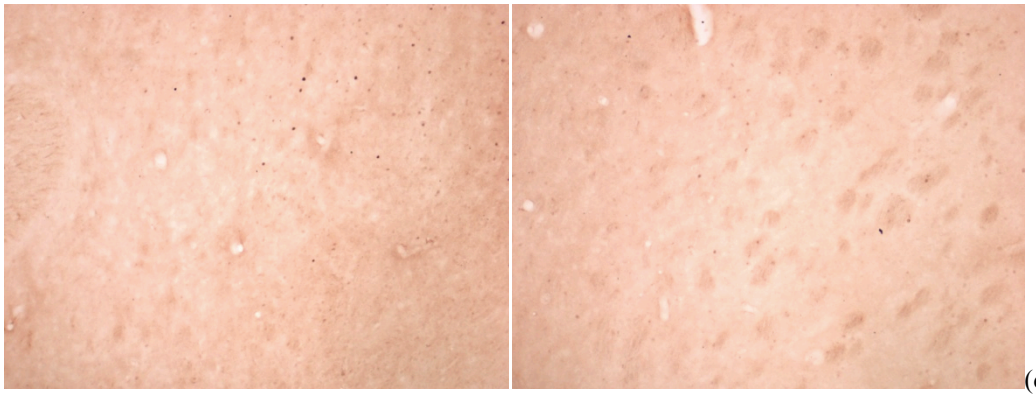

(d)

Figure 15: a) combination example (METH 0.75mg/kg and RO5203648 5mg/kg) NAC and DST clearly shows enhanced induction of cFos b) RO5203648 (5mg/kg) alone example of NAC and DST c) Meth (0.75mg/kg) alone example of NAC and DST d) Saline example of NAC and DST demonstrates low level of cFos expression.

The cFos density was clearly higher in the DST than the NAC especially in the group administered both RO5203648 and METH treatments.

3.3.Experiment 3 Results

This experiment examined the effects of RO503648 on METH self-administration and assessed the partial agonist's own abuse liability in the same context. The effects of RO5203648 were also tested on responding for natural reward.

3.3.1. Methamphetamine Self-administration

To investigate the effects of RO5203648 on METH intake, rats with a stable history of METH consumption (animals achieved consistent levels of METH intake during the training phase) were treated with different doses of the TAAR1 partial agonist (0, 3, and 10 mg/kg i.p.) 10 minutes before starting the self-administration session. An ANOVA was performed, with treatment group (saline or METH) as the independent variable and the number of reinforcements as the dependent variable. The experimental group that self-administered METH was significantly different from the control group that self-administered saline averaged across all sessions, $F(1,10) = 16.427$, $p = .0029$ with *post-hoc* test revealing significant results as well ($M = -10.944$, $SD = 6.109$, $p = .0029$) . There was a significant difference between the three doses of RO5203648, $F(2,10) = 8.935$, $p = .0020$. There was also a significant interaction between the different doses of the partial agonist and group (saline vs. METH) $F(2,10) = 7.163$, $p = .0051$. Scheffe's tests revealed significant differences between saline and the low dose of RO5203648 ($M = 7.545$, $SD = 5.248$, $p = .0046$) as well as

saline and the high dose of RO5203648 (M = 13.091, SD = 5.248, p<.001). There was also a significant difference between the low and high dose of RO5203648 (M = 5.545, SD = 5.248, p = .0373). These results imply that RO5203648 reduces METH intake dose-dependently as seen in *Figure 16.*

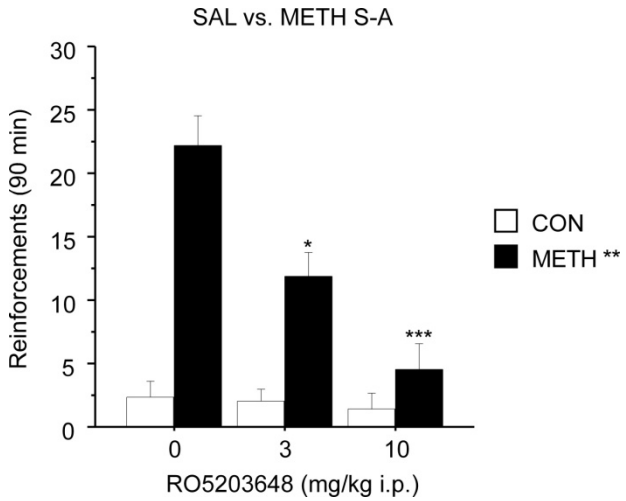

Figure 16: Mean deviation scores (±SEM) between saline and METH self-administration for each pre-treatment of RO5203648. This graph demonstrates RO5203648's dose-dependent reduction on METH self-administration, the graph also shows that the saline group is not affected by pre-treatment of RO5203648. *** indicates statistical significance of p<.001, ** indicates statistical significance of p<.01 and * indicates statistical significance of p<.05.

3.3.2. RO5203648 Substitution Self-administration

This experiment involved the TAAR1 partial agonist, RO5203648, being substituted for METH in the self-administration paradigm. Thus rats received infusions of RO5203648 at different doses (0, 0.25, 0.5 and 1.0 mg/kg/infusion) as the reinforcement resulting from a lever press. This was done to test RO5203648's reinforcing effects. After the RO5203648 substitution procedure a final test was introduced in which a small dose of METH (0.17 mg/kg/infusion, 3-fold weaker than the training dose) available on the lever to ensure that all animals were sensitive to changes in the reinforcing value of the self-administered solution, which was shown

to be the case. There was a significant difference between groups (saline vs. METH) $F(1,10) = 6.142$, $p = .0479$. Scheffe's tests also demonstrated this significant effect ($M = -19.900$, $SD = 19.648$, $p = .0479$). The difference between RO52034648 substitution doses and the small dose of METH was significant $F(4,10) = 3.062$, $p = .0359$. There was also a significant interaction between RO5203648 substitution and group $F(4,10) = 3.062$,$p = .0359$. Further results revealed significant differences between all RO5203648 substitution doses and the METH probe. The control for RO5203648 was significant at ($M = -23.875$, $SD = 17.400$, $p = .0036$), 0.25mg/kg ($M = -22.750$, $SD = 17.400$, $p = .0058$), 0.50mg/kg ($M= -25.000$, $SD = 17.400$, $p = .0022$), and the high dose (1.0mg/kg) at ($M = -22.625$, $SD = 17.400$, $p = .0061$). *Figure 17* demonstrates RO5203648's low reinforcing effects in comparison to the small METH dose.

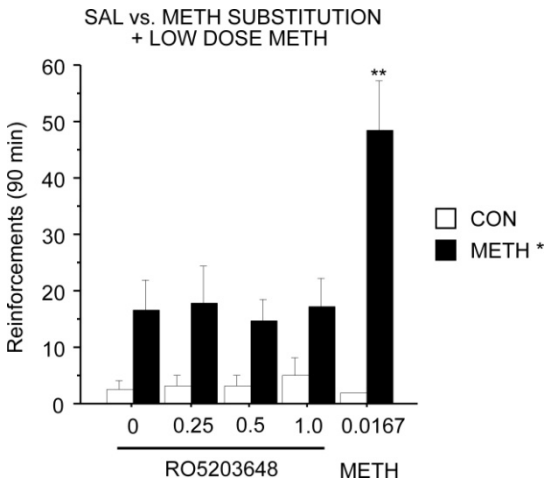

Figure 17: Mean deviation scores (±SEM) for overall group differences between saline and METH self- administration with RO5203648 substitute and low dose METH. This graph clearly shows that RO5203648 has low abuse liability in comparison to a probe test of METH. ** indicates statistical significance of $p<.01$ and * indicates statistical significance of $p<.05$.

This data critically demonstrates that self-administration of different doses of RO5203648 did not generate varying levels of responding, that is expected of a

reinforcing stimulant compound. However, METH did produce shifts in operant responding when self-administration doses were changed.

3.3.3. Saccharin Reinforcement

Lastly, in order to determine that the decrease in METH intake induced by RO5203648 was not caused by motor or motivational impairment, rats were trained to respond for saccharin and tested under the effects of RO5203648, given as a pre-treatment in the self-administration chambers. This tested the partial agonist's effects on rat's ability to respond to normal reward. An ANOVA was performed with RO5203648 treatment as the independent variable and the number of reinforcements from active lever presses was the dependent variable. It was found that RO5203648 did not significantly affect responding for natural reinforcement (p = .1621). The difference between saline and METH self-administration groups was also not significant when responding for saccharin (p = .4516). The interaction between groups and the different doses of RO5203648 was not significant (p = .9471). *Figure 18* shows these non significant findings.

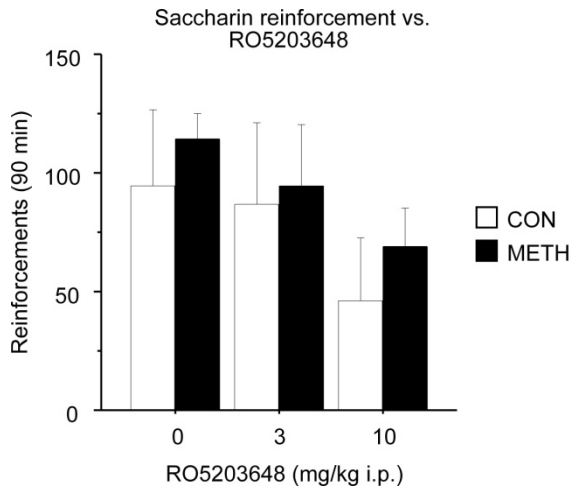

Figure 18: Mean deviation scores (±SEM) for saccharin reinforcement with RO5203648 pre-treatments. This shows the lack of effect RO5203648 has on natural reward although a slight decrease in responding can be seen at the high dose of RO5203648.

These results demonstrate that pre-treatment with RO5203648 does not affect responding for normal reward and that rats were not impaired as they were able to successfully lever press for saccharin.

5. Discussion

5.1. Summary of Results

The results for experiment 1 revealed that the TAAR1 partial agonist, RO5203648, can reduce METH induced locomotor stimulation in the open field paradigm. This provides a possible advantage for RO5203648 as an effective treatment in reducing METH-induced psychostimulant effects. RO5203648 also demonstrated its ability to attenuate METH-induced locomotor sensitization. Taken together these results support the initial hypothesis that the partial agonist may be effective in reducing METH-induced behaviours. However stimulus-response and impulsivity results were not as conclusive as METH-induced locomotor stimulation and sensitization. The stimulus-response task was not effectively mastered by the rats and therefore no firm conclusions could be drawn. The impulsivity task results showed that when presented with a delay rats are more likely to choose the immediate smaller reward rather than wait for the larger reward. Furthermore, previous history of drug exposure did not alter this pattern. However, due to limitations in the stimulus-response and impulsivity tasks it is not possible to conclude whether RO5203648 had any significant impact on these outcomes.

Experiment 2 results revealed that locomotor activity was significantly enhanced by the treatment of METH alone and RO5203648 treatment was not able to attenuate METH-induced locomotor stimulation. Administration of RO5203648 alone showed similar locomotor stimulation to the saline treated group. The findings from cFos analysis also showed that the treatment group that received RO5203648 and METH had significantly increased expression of cFos in both the NAC and DST compared to saline and RO5203648 alone treated groups. The hypothesis that

RO5203648 would attenuate cFos expression in the NAC and DST was not supported.

The last experiment, again demonstrated RO5203648's ability to positively attenuate METH-induced behaviours in the self-administration paradigm. The results showed that when rats are pre-treated with the partial agonist their responding for METH is significantly reduced, which supports the initial hypothesis. The behaviour observed when METH was substituted for RO5203648 also supports the research hypothesis as this suggests that rats did not actively seek self-administration of the partial agonist. This is further supported by the findings that rats significantly increased their responding for a lower dose of METH. Lastly, rats did not significantly reduce their response for a natural reward when pre-treated with RO5203648 thereby supporting the hypothesis that pre-treatment of RO5203648 would not deter the rat's ability to respond for saccharin.

5.1.1. Locomotor Stimulation and Sensitization

Increased activity is a well established side effect of psychostimulants, particularly METH, as both animal and human studies have demonstrated stimulants ability to elevate energy (Fujiwara et al, 1987; Strakowski et al, 1996; Stahl et al, 1997; Molitor et al, 1998; Rawson et al 2002; Cartier et al 2006, Shuto et al, 2006; Hall et al, 2008). This observation was also strongly apparent in the results of the present study. Rats administered with METH alone regardless of dose showed increased locomotor stimulation compared to saline treated rats. It is interesting to note that the low dose METH group produced significantly higher rates of locomotor activity than the high dose METH group. One possible explanation is the higher dose of METH manifested behaviourally as increased stereotypy rather than locomotor

activity. However, stereotypy was not measured in this experiment. Several studies have supported the claim that there is a reduction in locomotor activity resulting from higher rates of stereotypical behaviour after high doses of METH or chronic exposure to METH (Fujiwara et al, 1987; Lett, 1989; Hall et al, 2008). Therefore it is likely that rats treated with the high dose of METH in this experiment exhibited lower rates of locomotor stimulation due to enhanced stereotypical behaviour.

There was also a significant difference in the groups treated with the partial agonist alone in comparison to the METH alone groups. This finding implies that RO5203648 does not have locomotor enhancing properties typical of psychostimulant drugs. Rats administered with METH exhibited increased locomotor stimulation in relation to rats receiving RO5203648 alone. The high and low dose of RO5203648 alone showed similar locomotor activity to the saline treated rats. The interaction across all groups' demonstrated RO5203648 ability to dose-dependently decrease METH induced locomotor activity. All groups showed a significant reduction in locomotor activity over time. Specifically, METH treated rats across all sessions showed a significant difference in locomotor activity, again the low dose METH group showing higher stimulation than the high dose METH group. Previous studies examining other compounds for the treatment of psychostimulant addiction have generally failed to demonstrate a dose-dependent reduction of the effects of METH. For example, Szumlinski et al (2000) found that IBO and the synthetic IBO derivative, 18-MC did not attenuate METH induced locomotor activity and stereotypy. Rather both pre-treatments enhanced METH induced locomotor activity and produced quicker onset of stimulant effects, with METH effects lasting longer. A later study using DA D_1 agonist have found similar results for reducing METH-

induced locomotor activity and behavioural sensitization however this finding does not extend to cocaine sensitization. Further studies investigated a DA D_3 antagonist and its attenuating effects on both cocaine and METH (Xi et al., 2005; Higley et al., 2011). In the METH experiment these effects were only evident under progressive ratios of self-administration by reducing the break point. These reductions in METH's reinforcing effects are promising yet weak as the antagonist was not able to reduce fixed ratio METH self-administration. Ideally, a therapeutic approach to addiction would be effective in treating a wide range of psychostimulants (e.g. cocaine, METH, AMP) in all validated paradigms of animal tests that model human substance dependence.

Furthermore, locomotor activity within a session, averaged across all sessions, showed a significant decrease. This is likely due to an initial reduction in anxiety after becoming habituated to the environment after 60 minutes. In particular, METH treated rats showed a significant decrease in locomotor stimulation across the averaged session and this could be due to the rats habituating to METH effects. This could also be due to classical conditioning, as sufficient pairings between the drug and the testing environment could result in the initiation of the body's opponent process (Koob & Le Moal, 1997). Meaning, that exposure to the testing environment itself would be enough to start counteracting the effect of METH and thereby reduce locomotor activity. All treatment groups with RO5203648 showed a significant reduction in METH-induced locomotor activity across time, this finding suggests a therapeutic-like effect of the TAAR1 partial agonist. Crucially, rats treated with RO5203648 alone did not show enhanced locomotor behaviour, suggesting that RO5203648 does not act like a psychostimulant in the locomotor paradigm. This

further supports RO5203648's promise as potential target for METH addiction treatment.

The probe test of METH showed that behavioural sensitization occurred after chronic exposure to METH. Furthermore, the METH alone group's showed enhanced locomotor activity compared to saline which also suggests that rats previously exposed to METH became sensitized to a lower dose of METH. At a neural level, this could suggest a fundamental change in functioning of the reward systems associated with METH addiction (Robinson and Berridge, 1993, 2000; Wyvell & Berridge, 2001; Vanderschuren & Pierce, 2009). The results also revealed RO5203648 was able to effectively block METH-induced locomotor sensitization. However, there was an unexpected result in the sensitization test, where it appears that the partial agonist exhibited cross-sensitization, observed as an increase in METH-induced locomotor stimulation in groups treated with RO5203648 alone, compared to those treated previously with saline. It is important to assess possible cross sensitization effects of potential treatments. For example, Kuczenski and Segal (2002) found that Methylphenidate (Ritalin) did not cross sensitize to METH which supports its use as a medication for ADHD and other disorders. Therefore, these complex results suggest that chronic treatment with the partial agonist alone is able to produce lasting neuroadaptations that may be akin to those evoked by METH. Further neurobiological studies are needed to examine this possibility.

5.1.2. Stimulus-response and impulsivity

Previous research suggests that drug treated rats would be able to learn a stimulus-response task faster than saline treated rats due to quicker habit formation after drug administration (Nelson & Killcross, 2006). However, in this experiment

rats were unable to learn the stimulus-response task. Furthermore, the stimulus-response habit learning and impulsivity tasks did not effectively demonstrate RO5203648 therapeutic-like properties as the majority of the results were not significant. In the stimulus-response task there was a significant interaction between RO5203648 and METH groups. The group administered a high dose of METH in combination with a low dose of RO5203648 had more correct responses than the low dose of METH combined with RO5203648 and RO5203648 alone, which showed very similar response rates. It is difficult to conclude from these results whether the RO5203648 had any therapeutic-like effects on METH-induced deficits in stimulus-response habit learning as these rats performed the task just above chance, therefore not providing enough power to detect differences and support any valid conclusions. This limitation is further discussed in the section *methodological limitations.* The impulsivity task revealed a significant interaction between times of delay when responding for larger rewards. This supported, as expected, that rats were more likely to choose the larger reward when there was no delay but when a delay was introduced their ability to wait for the larger reward decreased. Due to results not being significant, conclusions on RO5203648's ability to inhibit impulsive choice in chronically METH treated rats cannot be confidently reached. This issue is also addressed in the section *methodological limitations*.

5.1.3. Locomotor activity and cFos expression in NAC and DST

The locomotor activity findings for this experiment refer to RO5203648's effects on acute administration of METH rather than chronic effects. This means that RO5203648 seems to potentiate locomotor activity in combination with METH after acute administration whereas after chronic exposure to METH the pre-treatment of

RO5203648 successfully reduces METH-induced locomotor stimulation, which was evident in experiment 1. However, in experiment 1 RO5203648 also attenuated METH-stimulated locomotor activity after the first (acute) treatment, thus producing a discrepancy. The difference in the results of experiment 1 and experiment 2 could be due to rat's difference in age as experiment 2 rats were much older than experiment 1 thereby affecting the expression of locomotor activity as the younger rats had higher rates of locomotion compared to the older rats. Expression of the immediate early gene cFos in the NAC and DST were measured after acute administration of saline, METH, RO5203648, or METH and RO5203648 combination. Results showed that rats treated with the combination of RO5203648 and METH had enhanced cFos expression in both the NAC and DST compared to those treated with RO5203648 alone and saline. METH also demonstrated increased cFos expression in both brain regions however it was not significantly different to the other treatment groups. This finding is supported by several past studies investigating METH's effects on cFos protein expression. Umino et al (1995) and Thiriet et al (2001) both demonstrated that METH induces cFos expression in DA rich brain regions, including the NAC and DST. The partial agonist itself did not display the ability to stimulate cFos in either brain region. These results suggest that RO5203649 does not induce cFos expression in the NAC and DST, yet when it is given in conjunction with METH it appears to have an enhancing effect in these brain regions which is not consistent with its ability to attenuate METH-stimulated behaviour. This experiment only measured the acute effects of RO5203648 on both locomotor activity and cFos expression therefore, it may be that RO5203648 would have attenuating effects on both locomotor activity and cFos expression after chronic exposure as RO5203648 was found to attenuate METH-induced locomotor activity in experiment 1.

5.1.4. METH Self-administration and RO5203648 substitution

Firstly, as expected, the experimental group that received METH showed significantly enhanced responding for infusions due to METH's reinforcing properties compared to the control group that received saline (Molitor et al, 1998; Julien, 2001; Rawson et al 2002; Topp et al, 2002; Cartier et al 2006; Wu et al, 2007). Again, as predicted, RO5203648 dose-dependently reduced METH self-administration, demonstrating the partial agonist's potential to effectively treat METH addiction. The control group responses remained relatively stable when pre-treated with RO5203648. Previous studies aiming at reducing METH self-administration have only been partially successful. For example, Higley et al's (2011) approach to a potential METH treatment with the DA D_3 receptor antagonist SB-277011A found no reducing effects on METH self-administration, yet it was able to lower the break point for METH self-administration. The antagonist was also able to inhibit METH reinstatement. These findings provided some promise for DA D_3 receptor antagonists as potential medication for METH addiction. However, SB-277011A was not able to reduce METH self-administration (Higley et al, 2011).

Animal studies of modafinil have been successful in METH relapse models (Yahyavi-Firouz-Abadi & See, 2009) yet in clinical trials of modafinil were unsuccessful in treating METH dependent participants (Heinzerling et al, 2010). Clinical trials of bupropion were also unsuccessful (Shoptaw et al, 2008). This suggests that targeting DA and NE systems does not sufficiently reduce METH's reinforcing properties. TAAR1-based compounds provide a new way of approaching the development of METH addiction through their ability to regulate DAT activity in

brain regions known to be involved in psychostimulant abuse including neurons of the VTA (Lindemann & Hoener, 2005).

The suitability of RO5203648 development as a therapeutic treatment for METH addiction was further supported by the substitution experiment. When RO5203648 was substituted in the self-administration paradigm there was a significant difference in self-administration between METH and control groups. The aim of this experiment was to assess the partial agonist's drug abuse liability by testing whether rats would freely self-administer RO5203648. The findings showed a residual effect of responding in the control condition (saline with 20% DMSO). However, the number of reinforcements for each subsequent RO5203648 dose did not significantly differ from the residual effect. This result was further supported by a probe test where all rats received a small dose METH that resulted in a significant increase in the rate of reinforcement. This data demonstrates that even a small dose of METH had more reinforcing effects than RO5203648. In other words, the rats were considerably more motivated to respond for METH than for RO5203648.

In the last set of experiments the effects of RO5203648 on natural reward were tested. The results supported the hypothesis that RO5203648 does not negatively affect the rat's ability to respond for natural reward. Similar results have been found for other potential treatments of psychostimulant addiction in past studies. Ferragud et al (2009) and Velazquez-Sanchez et al (2010) investigated the BZT derivate, AHN-1055, on cocaine and AMP induced behaviours, respectively. AHN-1055 was able to dose-dependently decrease cocaine and AMP self-administration and did not exhibit reinforcing effects of its own. However, AHN-1055 did not reduce intake of sucrose in an operant task, suggesting that the decreasing effects of this analogue on cocaine

and AMP self-administration were not due to impaired motor or motivational processes. METH, amongst other psychostimulants, is an agonist of TAAR1 (Bunzow et al., 2001; Grandy et al., 2007) and these findings support the idea that partial agonists play a role in the reinforcing effects and maintenance of METH addiction. Therefore by targeting TAAR1 based medications for development is promising. The present study demonstrates similar effects of the partial TAAR1 agonist on METH self-administration.

5.2.Methodological strengths

There are a number of methodological strengths in this study. Firstly, the use of rats to investigate the newly developed TAAR1 partial agonist allows for several different models of addiction to be tested. The use of recognised animal models of addiction and cognitive tasks, such as locomotor stimulation and sensitization, impulsivity and stimulus-response, cFos expression, and lastly self-administration provide a wide scope for examining the effects of a potential anti-addiction medication. These paradigms have been used for many years and have provided a sound base to assess animal behaviour in relation to humans. It is also important to note that the present study looks specifically at METH addiction. In human studies involving addiction it is often hard to rule out any contributing effects of other substances an individual may use. Such poly drug use of people who typically abuse drugs is wide ranging and may include cigarettes, alcohol, stimulants or opiates. When examining drug use in humans self-reports can be unreliable and inconclusive. Animal studies offer a more structured approach to studying addiction and allow conclusive evidence of drug effects on behaviour and cognitive functioning. Possible treatments for drug addiction need to be tested in animal models of addiction before

any clinical data can be obtained. This is an essential step in developing an effective therapeutic approach to many illnesses.

5.3. Methodological limitations

There are several limitations that became apparent throughout these studies. Firstly, the implications of animal research for possible human medications should be treated with caution. These experiments provide pre-clinical evidence supporting the use of the TAAR1 partial agonist, RO5203648, for the treatment of METH addiction. However, there is currently not enough information to conclude this compound is safe for human consumption. Additional animal studies are needed to further examine its potential effects before considering a clinical trial in humans.

The strain of rat was another important factor in these experiments. Both experiments 1 and 2 used the PVG hooded strain of rats. This particular strain seemed to be hypersensitive to METH as previously tested doses of METH (3 mg/kg) were potentially lethal. These rats were also very slow to learn their assigned tasks. For example, the stimulus-response task was not successful because the rats were still performing at chance levels over 500 trials. With more extended training these rats may have been able to complete the task; however time constraints were an issue making it impractical to continue the task.

The sample sizes across all experiments were somewhat small, in experiment 1 typical group sizes were 6 rats per treatment condition. Experiment 2 only had 4 rats per group, with the exception of the METH combined with RO5203648 group which had 5. Lastly, experiment 3 had 10 rats in the experimental group that self administered METH whereas the control group only had 2 surviving rats. Although

significant results were obtained future studies would benefit from larger group sizes to provide stronger statistical power and support for results.

Furthermore the delay between the last treatment of RO5203648 and METH and the beginning of testing for impulsivity was long (waiting for rats to learn stimulus-response task). Thus, the full effect of these pharmacological treatments on impulsive behaviour may not have been apparent. To combat this limitation, a small dose of METH was administered before the tests of impulsivity. However, long lasting effects of previously administered treatments were not found on impulsive choice. The addition of low METH doses with the pre-treatment of RO5203648 could have been beneficial (same treatment rats received in the locomotor paradigm) so that stronger interactions between RO5203648 and METH for both stimulus response and impulsive behaviour could have been concluded.

In summary, the limitations of these experiments do not subsequently diminish the importance of the results obtained. These experiments conclusively show that RO5203648 holds potential as a future therapeutic approach to METH addiction.

5.4.Implications

These studies are the first experimental behavioural research that has been conducted on the TAAR1 partial agonist, RO5203648 for the specific treatment of METH addiction. Research involving TAAR1 as a novel receptor target for medication in drug addiction is supported by several studies demonstrating its role in monoamine systems and psychostimulant activation. *In vitro* studies involving TAAR1 have shown regulation of monoaminergic transporters, specifically the DAT. Xie and Miller (2009) have demonstrated that METH causes a TAAR1-dependent

inhibition of DA uptake and DA release through the DAT. This regulation of DAT activity, mediated by TAAR1, may serve as an important process in psychostimulant mechanisms of action, particularly in psychostimulant neuroadaptations (Xie & Miller, 2009). Further support for TAAR1 involvement and development in possible psychostimulant treatment has been found in TAAR1 knockout mice. Deficits in prepulse inhibition are induced by psychomotor stimulants and this study showed that TAAR1 knockout mice also exhibited this deficit and showed enhanced sensitivity to AMP suggesting TAAR1 mediates the effects of psychostimulant drugs (Wolinsky et al, 2007). Recent *in vivo* studies using selective TAAR1 agonists on cocaine have also found dose-dependent attenuation of cocaine-induced locomotor activity yet this agonist was not able to alter cocaine-induced CPP (Revel et al., 2011). Furthermore, TAAR1's ability to mimic AMP-like molecules, alter DA transmission, and activate K+ channels provides sufficient support for TAAR1-related treatments to be developed in relation to the neuroadaptations on the DA system that are produced by drugs (Bradaia et al, 2009).

The present study adds to literature involving TAAR1 medication development as it demonstrated the possible therapeutic effects of this newly developed compound through widely accepted animal models of METH addiction. The animal models of addiction used allow further understanding of the mechanisms behind psychostimulant addiction. This can be seen through RO5203648 effects on METH induced behaviour, as the compound was able to reduce locomotor stimulation, sensitization and self administration which imply that TAAR1 specific pathways/regions are involved in the development or at least the maintenance of these METH-related behaviours. By developing an effective treatment for this specific type

of addiction reductions in health problems, aggression and crime may decrease as a result. These findings have implications not only for New Zealand but worldwide.

5.5.Future Research

Future research further investigating TAAR1 partial agonist, RO5203648, is warranted. The first step towards expanding the literature on this potential treatment of psychostimulant addiction should involve addressing the limitations of these experiments outlined previously in *methodological limitations*. Increasing sample sizes of experimental and control groups will offer more power for statistical significance and stronger conclusions can be drawn from results. The strain of rat to be used in drug related experiments should also be considered as some strains have been shown to be hypersensitive to psychostimulants and other drugs. The present studies suggest that the Long Evans strain of rat performed at a superior level compared to other strains used in these experiments. This was evident in the Long Evans ability to quickly learn their assigned tasks and handle the doses of METH.

Replication of the present research will provide further evidence for RO5203648 effects on psychostimulant dependence. Other tools to study TAAR1 function should be considered, including full agonists, antagonist and animals with deletion of the TAAR1 gene. The use of additional animal models of addiction would enhance understanding of the mechanisms behind the interactions between TAAR1 partial agonist and drugs of abuse such as METH. Behavioural models for future research should aim to include drug discrimination, conditioned place preference, and relapse models of addiction. At the neurobiological level the development of full TAAR1 agonists and antagonists will also provide in depth look into the neural pathways implicated in psychostimulant addiction. One way to truly understand the role

TAAR1 plays in stimulant dependence is through the development of TAAR1 knockout animals. Investigation into the neurochemical interactions between the DA systems and TAAR1 will also add breadth to understanding the underlying properties of potential medications. Lastly, future research should eventually aim to conduct clinical trials on TAAR1-based medications for psychostimulant addiction, only after all effects of these compounds are fully characterised in animal models.

5.6.Conclusions

Drug use and abuse contributes to a range of issues, for the individual addicted and also for the wider community. Action to reduce rates of drug dependence is warranted through the development of therapeutic agents. However, the development of such effective medications for psychostimulant addiction has been difficult. Behavioural and social programmes aimed at psychostimulant dependence are only partially effective therefore pharmacological treatment would aid recovery from addiction. Thereby targeting the newly discovered TAAR1 receptors in relation to drug addiction provides a promising approach to medication development due to their ability to regulate monoamine systems and be activated by psychostimulants. This study clearly outlines RO5203648's potential use as a treatment for METH addiction through its capacity to reduce METH-induced locomotor activity and sensitization as well as METH self-administration without exhibiting any addictive properties of its own. It also provides further support in targeting TAAR1 medication development for drug dependence. Additional studies are needed to further understand the mechanisms behind RO5203648's complex effects on behaviour and neural expression in relation METH addiction.

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Appendix A

Open field apparatus set up in windowless room

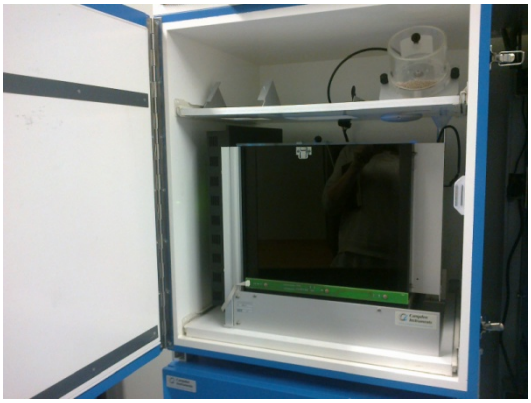
Width: 50 cm

Length: 40 cm

Height: 35 cm

Appendix B

Bussey-Saksida touch-screen chambers set up in windowless room.

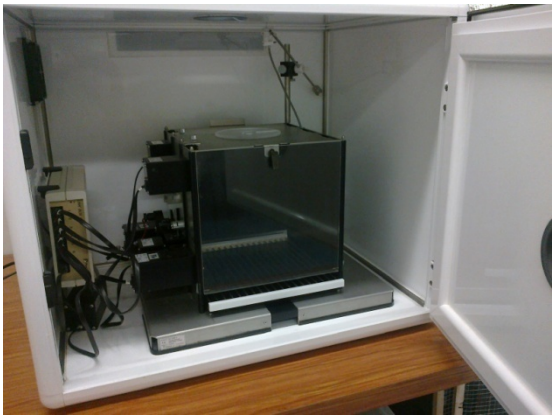


Inside chambers

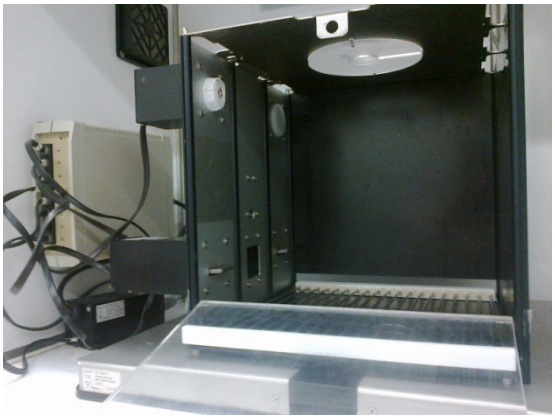
Touch screen at one end and the food well at the other end of chamber.

Appendix C

Self-administration chamber set up in windowless room.



Inside the operant chamber.

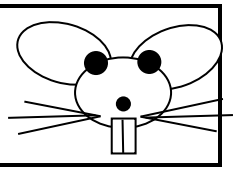


2 retractable levers and reward well in between the levers. Stimulus light and speaker are above levers.

Appendix D

POST OPERATIVE EVALUATION

Name **AEC #** **IDAO** **Surgeon/s:** _____

Animal ID #	Strain:	
Colour (spray)		
Date of surgery		

Date							
Time							
Physical observations							
B. A. R							
Faeces							
Breathing *							
Eating							
Drinking							
Suture line							
Sutures okay							
Observer Initials							

* **N** = normal, **L** = laboured, **R** = rapid, **S** = shallow

Comments: